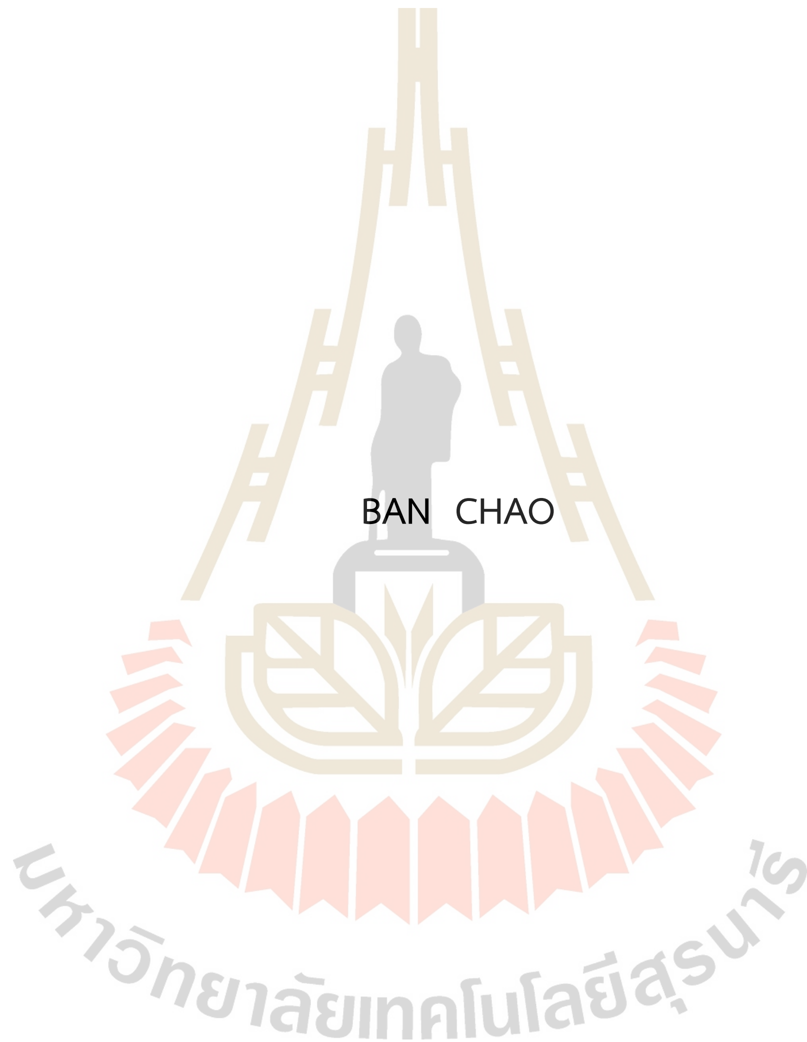
ASSESSMENT OF HEAT KILLED *LIMOSILACTOBACILLUS INGLUVIEI* C37 AS A POSTBIOTIC HEALTH SUPPLEMENT FOR CALVES



BAN CHAO

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy Program in Animal Technology and Innovation

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การประเมินผลของ *Limosilactobacillus ingluviei* C37 ที่ถูกทำให้ตาย ด้วยความร้อนเพื่อเป็นสารเสริมสุขภาพโพสไบโอติกสำหรับลูกโค

นายบัน เชาว์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์
มหาวิทยาลัยเทคโนโลยีสุรนารี
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ASSESSMENT OF HEAT KILLED *LIMOSILACTOBACILLUS INGLUVIEI* C37 AS A POSTBIOTIC HEALTH SUPPLEMENT FOR CALVES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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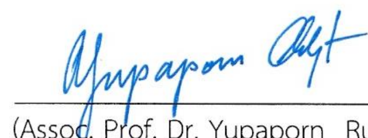

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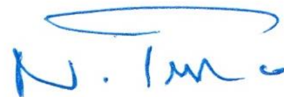

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มหาวิทยาลัยเทคโนโลยีสุรนารี

บัน เชาว์ : การประเมินผลของ *Limosilactobacillus ingluviei* C37 ที่ถูกทำให้ตายด้วยความร้อนเพื่อเป็นสารเสริมสุขภาพโพสไบโอติกสำหรับลูกโค (ASSESSMENT OF HEAT KILLED *LIMOSILACTOBACILLUS INGLUVIEI* C37 AS A POSTBIOTIC HEALTH SUPPLEMENT FOR CALVES) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร. พิพัฒน์ เหลืองลาวัณย์, 120 หน้า.

คำสำคัญ: สารต้านอนุมูลอิสระ/ลูกโคหย่านม/ประสิทธิภาพการเจริญเติบโต/โพสไบโอติก/ทรานสคริปโตม

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของโพสต์ไบโอติกจาก *Limosilactobacillus ingluviei* C37 ที่ถูกทำให้ตายด้วยความร้อนต่อประสิทธิภาพการเจริญเติบโต ความสามารถในการต้านอนุมูลอิสระของพลาสมา ทรานสคริปโตมของตับและลำไส้เล็กส่วนกลางของลูกโคในช่วงหย่านม

ลูกโคนมเพศผู้พันธุ์โฮลสไตน์จำนวน 14 ตัว (อายุ 5.71 ± 1.14 วัน) ถูกแบ่งเป็นบล็อกโดยน้ำหนักตัว (37.34 ± 3.19 ก.ก. และ 28.83 ± 2.92 ก.ก.) และทำการแบ่งกลุ่มแบบสุ่มเป็น 2 กลุ่ม (กลุ่มละ 7 ตัว) กลุ่มควบคุมได้รับอาหารพื้นฐานและกลุ่มการทดลองได้รับอาหารพื้นฐานร่วมกับ *Limosilactobacillus ingluviei* C37 ที่ถูกทำให้ตายด้วยความร้อน 1 กรัม/วัน (10^8 CFU/g) ลูกโคทั้งหมดได้รับการหย่านมภายในวันที่ 89 ของการทดลอง ผลการทดลองแสดงให้เห็นว่าการเสริมโพสต์ไบโอติกช่วยปรับปรุงประสิทธิภาพการใช้อาหารในช่วงการทดลองวันที่ 32 - 89 และตลอดระยะเวลาการทดลอง (วันที่ 1 - 89) ($P < 0.05$) เมื่อเปรียบเทียบกับกลุ่มควบคุม พบว่าโพสต์ไบโอติกสามารถลดระดับ globulin, total protein, neutrophil (Neu) และ ratio of neutrophil to lymphocyte (NLR) ($P < 0.05$) นอกจากนี้ระดับ serum urea nitrogen (BUN), triglyceride (TRIG), และ cholesterol (CHOL) มีแนวโน้มลดลง ($P < 0.1$) เมื่อเสริมด้วยโพสต์ไบโอติก การให้โพสต์ไบโอติกทำให้ระดับ cortisol และ malondialdehyde (MDA) ลดลง และเพิ่มระดับ catalase (CAT) ($P < 0.05$) ในขณะที่มีแนวโน้มว่า glutathione peroxidase (GPX) level และ 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging capacity เพิ่มขึ้น ($P < 0.1$)

การวิเคราะห์ทรานสคริปโทมของตับระบุ differentially expressed genes (DEGs) ได้ 33 รายการ ประกอบด้วย 16 upregulated DEGs เช่น Endothelial lipase (LIPG), Peroxisomal Acyl-CoA oxidase 1 (ACOX1), Solute carrier family 27 member 6 (SLC27A6) และ 17 downregulated DEGs เช่น Family with sequence similarity 107 member A (FAM107A), 4-Hydroxy-2-oxoglutarate aldolase 1 (HOGA1), Farnesyl diphosphate synthase (FDPS) การวิเคราะห์ Kyoto Encyclopedia of Genes and Genomes (KEGG) ระบุได้ 11 significant pathways รวมถึง PPAR signaling pathway และ Pentose phosphate pathway

การวิเคราะห์ทรานสคริปโตมิกของลำไส้เล็กส่วนกลางระบุ DEGs ได้ 76 รายการ โดยมีการ upregulation of genes ที่เกี่ยวข้องกับ fatty acid metabolism (FABP1), intestinal barrier function (B4GALNT2), และ detoxification (GSTA1) อย่างมีนัยสำคัญ ควบคู่ไปกับ downregulation ของ immune response regulation (FCRLA, FCRL4) การวิเคราะห์ Gene Ontology (GO) และ Kyoto Encyclopedia of Genes and Genomes (KEGG) เน้นย้ำถึงการเสริมประสิทธิภาพใน pathways ที่เกี่ยวข้องกับ Glutathione metabolism, Drug metabolism, และ Vitamin digestion

โดยภาพรวม การศึกษานี้บ่งชี้ว่าการเสริม *Limosilactobacillus ingluviei* C37 ที่ถูกทำให้ตายด้วยความร้อน สามารถปรับปรุงประสิทธิภาพการใช้อาหาร เพิ่มสถานะสารต้านอนุมูลอิสระในพลาสมา และปรับภูมิคุ้มกันในลูกโคในช่วงหย่านม ข้อมูลทรานสคริปโตมิกยังชี้ให้เห็นถึงประโยชน์ต่อ metabolism, epithelial integrity และ detoxification อีกด้วย ผลการวิจัยเหล่านี้ชี้ให้เห็นว่าโพสไบโอติกอาจใช้เป็นกลยุทธ์ทางโภชนศาสตร์ที่สามารถบรรเทาความเครียดจากการหย่านมและส่งเสริมสุขภาพของลูกโค

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา________
ปีการศึกษา 2567 ลายมือชื่ออาจารย์ที่ปรึกษา________

BAN CHAO : ASSESSMENT OF HEAT KILLED *LIMOSILACTOBACILLUS INGLUVIEI* C37 AS A POSTBIOTIC HEALTH SUPPLEMENT FOR CALVES. THESIS ADVISOR : ASST. PROF. PIPAT LOUNGLAWAN, Ph.D., 120 PP.

Keyword: Antioxidant/Calf weaning/Growth performance/Postbiotic/Transcriptome

This study aimed to investigate the effects of postbiotic from heat-killed *Limosilactobacillus ingluviei* C37 on growth performance, plasma antioxidant capacity, liver and jejunal transcriptome of calves during weaning.

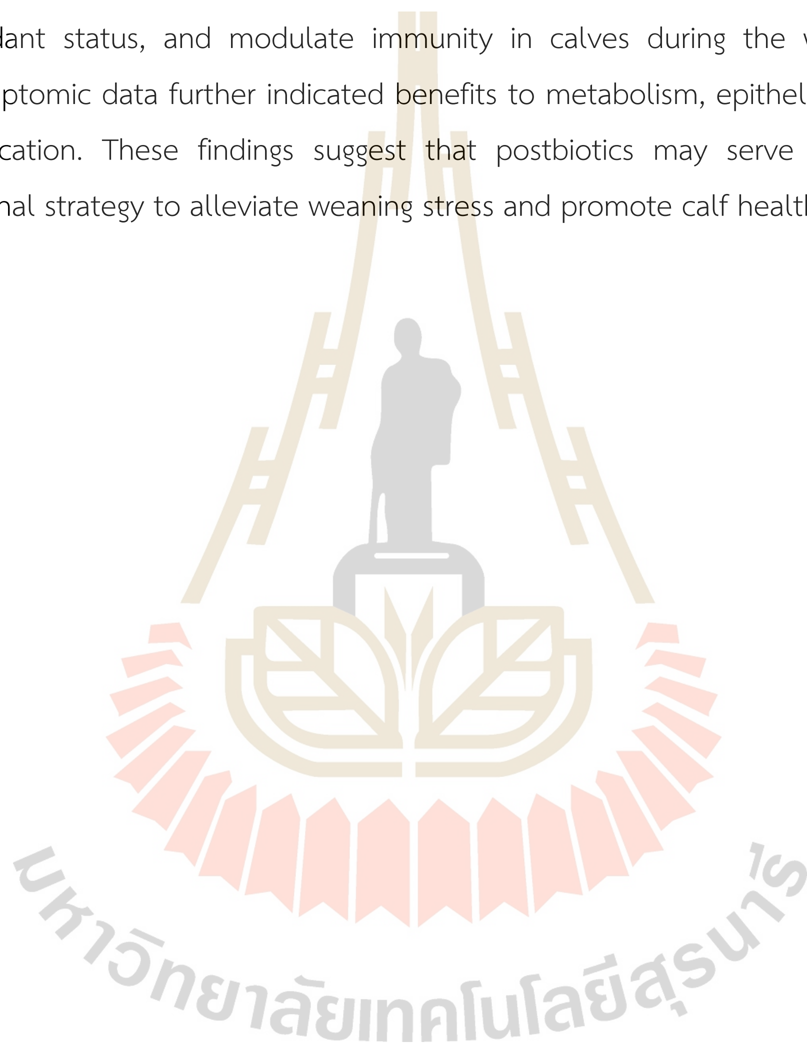
Fourteen Holstein bull calves (age: 5.71 ± 1.14 days) were blocked by body weight (BW) (37.34 ± 3.19 kg, and 28.83 ± 2.92 kg; mean ± SD) and randomly allocated into two groups (n = 7 per group): a control group receiving a basal diet (CON), and a treatment group feeding with 1 g/d of heat-killed *Limosilactobacillus ingluviei* C37 (10^8 CFU/g) (TRT). All calves were completely weaned by day 89. The results showed that postbiotic supplementation improved feed efficiency at day 32-89, and the overall period (day 1-89) ($P < 0.05$). Compared to the CON group, postbiotic reduced globulin, total protein, neutrophil (Neu) and the ratio of neutrophil to lymphocyte (NLR) levels ($P < 0.05$). Additionally, the serum urea nitrogen (BUN), triglyceride (TRIG), and cholesterol (CHOL) levels tended to decrease ($P < 0.1$) with postbiotic supplementation. Feeding postbiotic led to a reduction in cortisol and malondialdehyde (MDA) and increased catalase (CAT) levels ($P < 0.05$), while there was a tendency for increased glutathione peroxidase (GPX) levels and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging capacity ($P < 0.1$).

Liver transcriptome analysis identified 33 differentially expressed genes (DEGs), including 16 upregulated DEGs such as Endothelial lipase (LIPG), Peroxisomal Acyl-CoA oxidase 1 (ACOX1), Solute carrier family 27 member 6 (SLC27A6), and 17 downregulated DEGs such as Family with sequence similarity 107 member A (FAM107A), 4-Hydroxy-2-oxoglutarate aldolase 1 (HOGA1), Farnesyl diphosphate synthase (FDPS). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified 11 significant pathways, including the PPAR signaling pathway and Pentose phosphate pathway.

Jejunal transcriptomic analysis identified 76 DEGs, with significant upregulation of genes involved in fatty acid metabolism (FABP1), intestinal barrier function

(B4GALNT2), and detoxification (GSTA1), alongside downregulation of immune response regulation (FCRLA, FCRL4). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses highlighted enrichment in pathways related to Glutathione metabolism, Drug metabolism, and Vitamin digestion.

Overall, the present study indicated that dietary supplementation with heat-killed *Limosilactobacillus ingluviei* C37 could improve feed efficiency, enhance plasma antioxidant status, and modulate immunity in calves during the weaning period. Transcriptomic data further indicated benefits to metabolism, epithelial integrity, and detoxification. These findings suggest that postbiotics may serve as a promising nutritional strategy to alleviate weaning stress and promote calf health.



School of Animal Technology and Innovation Student's Signature__________
Academic Year 2024 Advisor's Signature__________

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On finishing the last word of my thesis, I see a heart filled with gratitude beating inside my chest. All things arise from causes, all phenomena are bound by affinity. The completion of this thesis is not the work of one alone. Looking back on the years of solitary study, my heart is filled with emotion. I cannot help but commit these words to paper as a token of gratitude.

To my advisor, Asst. Prof. Dr. Pipat Lounglawan, my deepest appreciation. Your wisdom, generosity, and belief in me have illuminated my path more than words can convey. Throughout this journey, your guidance has extended beyond the academic realm, providing me with not just knowledge, but invaluable mentorship and steadfast support.

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This work would not have been possible without the quiet strength and understanding of my family, whose love has been my constant source of comfort and motivation.

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มหาวิทยาลัยเทคโนโลยีสุรนารี

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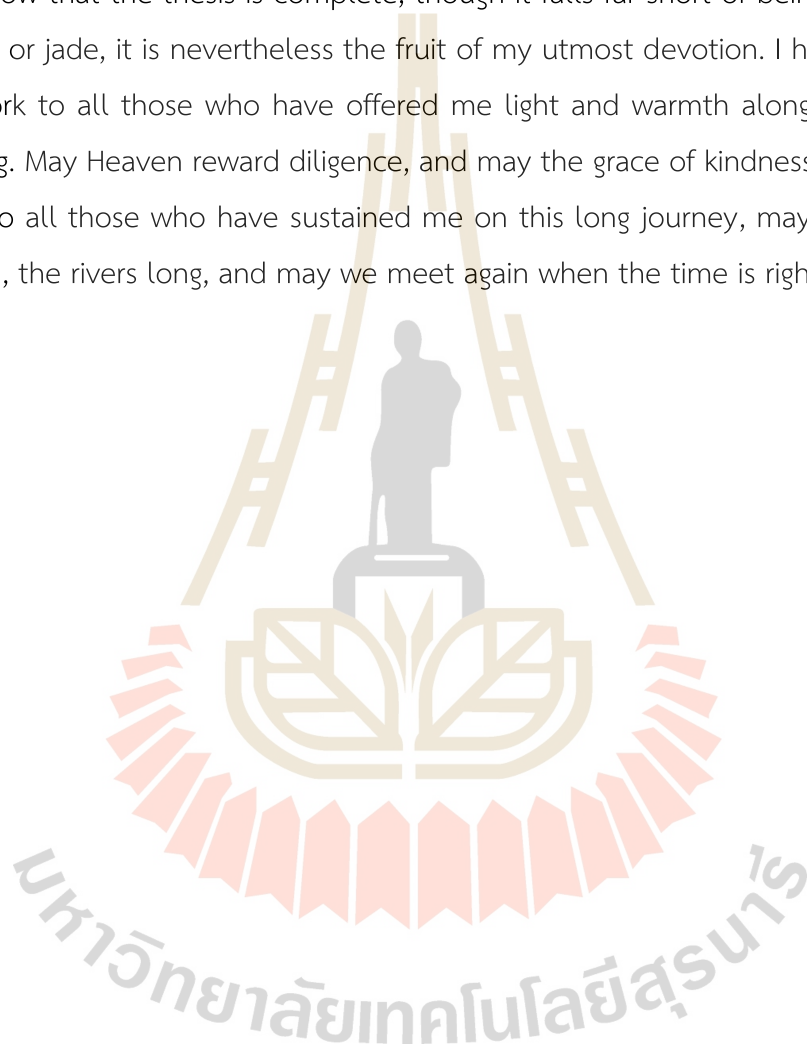
Now that the thesis is complete, though it falls far short of being called a work of gold or jade, it is nevertheless the fruit of my utmost devotion. I humbly dedicate this work to all those who have offered me light and warmth along my journey of learning. May Heaven reward diligence, and may the grace of kindness endure.

To all those who have sustained me on this long journey, may the mountains be high, the rivers long, and may we meet again when the time is right.

Ban Chao



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LIST OF ABBREVIATIONS

%	=	Percentage
°C	=	Degree celsius
ADF	=	Acid detergent fiber
ACOX1	=	Acyl-CoA oxidase 1
ADG	=	Average daily gain
ALT	=	Alanine aminotransferase
AOAC	=	Association of offcial analytical chemists
AST	=	Aspartate aminotransferase
B4GALNT2	=	Beta-1,4-n-acetyl-galactosaminyltransferase 2
BP	=	Biological processes
BUN	=	Serum urea nitrogen
BW	=	Body weight
CAT	=	Catalase
CBC	=	Complete blood count
CC	=	Cellular components
CFS	=	Cell free supernatant
CFU	=	Colony forming unit
CHOL	=	Cholesterol
CON	=	Control
CP	=	Crude protein
d	=	Day
DEG	=	Differential gene expression
dL	=	Deciliter
DM	=	Dry matter
DNA	=	Deoxyribonucleic acid
DPPH	=	2,2-diphenyl-1-picrylhydrazyl radical
ELOVL6	=	Elongation of very long chain fatty acids protein 6
EXTL1	=	Exostosin like glycosyltransferase 1

LIST OF ABBREVIATIONS (Continued)

FABP1	=	Fatty acid binding protein 1
FAM107A	=	Family with sequence similarity 107 member A
FC	=	Fold change
FCRL4	=	Fc receptor like 4
FCRLA	=	Fc receptor like a
FDPS	=	Farnesyl diphosphate synthase
g	=	Gram
GAPDH	=	Glyceraldehyde-3-phosphate dehydrogenase
GEO	=	Gene expression omnibus
GLM	=	General linear model
GLU	=	Glucose
GLYCTK	=	Glycerate kinase
GO	=	Gene ontology
GPX	=	Glutathione peroxidase
GSTA1	=	Glutathione s-transferase alpha 1
HCT	=	Hematocrit
HGB	=	Hemoglobin
HOGA1	=	4-Hydroxy-2-oxoglutarate aldolase 1
KEGG	=	Kyoto encyclopedia of genes and genome
kg	=	Kilogram
L.	=	Limosilactobacillus ingluviei C37
LIC37	=	Lactobacillus
Lymp	=	Lymphocyte
m	=	Miter
MDA	=	Malondialdehyde
MDMI	=	Milk replacer intake base on dry matter
MF	=	Molecular functions
min	=	Minute
mL	=	Milliliter

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LIST OF ABBREVIATIONS (Continued)

MR	=	Milk replacer
N	=	Nitrogen
NCBI	=	National center for biotechnology information
NDF	=	Neutral detergent fiber
Neu	=	Neutrophil
NLR	=	The ratio of neutrophil to lymphocyte
NLR	=	The ratio of neutrophil to lymphocyte
nm	=	Nanometer
NMDS	=	Non metric multidimensional scaling
ONECUT2	=	One cut homeobox 2
OTU	=	Operational taxonomic unit
PAX5	=	Paired box 5
PAX9	=	Paired box 9
PC	=	Principal components
PCoA	=	Principal co-ordinates analysis
PCR	=	Polymerase chain reaction
qPCR	=	Quantitative polymerase chain reaction
RBC	=	Red blood cell count
rDNA	=	Ribosomal deoxy ribonucleic acid
rRNA	=	Ribosomal RNA
SD	=	Standard deviation
SDMI	=	Starter intake base on dry matter
SEM	=	Standard error of mean
SLC27A6	=	Solute carrier family 27 member 6
TAOC	=	Total antioxidant capacity
TDMI	=	Total dry matter intake
TRIG	=	Triglyceride
WBC	=	White blood cell count
µg	=	Microgram

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LIST OF ABBREVIATIONS (Continued)

µL	=	Microliter
µmol	=	Micromole

CHAPTER I
INTRODUCTION

1.1 Introduction

Weaning is one of the more challenging and stressful events in dairy calves (Agustinho et al., 2024). The abrupt separation from milk, maternal contact, and a shift toward solid feed can induce a cascade of physiological and metabolic changes, collectively known as weaning stress (Weary et al., 2008). This condition is often characterized by elevated cortisol levels, impaired antioxidant defenses, compromised liver function, and disturbances in gut development and immunity (Kim et al., 2011; Ballou, 2012; Meale et al., 2017). For example, Lynch et al. (2010) reported that abrupt weaning causes a temporary impairment in calf immune function, marked by increased neutrophils and diminished phagocytic capacity. In addition, rapid dietary changes during weaning can impair lower gut function and reduce energy intake, ultimately leading to growth depression (Steele et al., 2017). Therefore, identifying effective nutritional strategies to alleviate weaning stress is of great significance for improving calf health and welfare.

For decades, probiotics have been widely used in animal feed to enhance growth performance and mitigate oxidative stress. However, the widespread presence of antibiotic resistance genes in these strains, and the demonstrated ability for these genes to transfer between organisms (Marteau and Shanahan, 2003), casts a long shadow over their suitability for continued use as live bacteria in future nutritional strategies. Offering a compelling alternative to traditional probiotics, postbiotics are defined as a “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host” (Salminen et al., 2021), which could enhance host antioxidant capacity and immunity, improve growth performance (Jalali et al., 2024; Monika et al., 2024). Recent studies have shown that dietary supplementation with heat-killed Lactobacillus postbiotics confers various health benefits in animals. For example, Xia et al. (2024) reported that postbiotics derived from heat-killed *L.*

acidophilus improved feed efficiency, apparent digestibility, and serum antioxidant capacity in rabbits. Similarly, Arellano-García et al. (2023) found that heat-inactivated *L. rhamnosus* GG enhanced liver antioxidant capacity (GPx, GSH), while reducing TNF-α in rats fed a high-fat, high-fructose diet. Additionally, Shu et al. (2024) showed that postbiotic supplementation in weaned piglets increased body weight gain, improved serum antioxidant status, and modulated lipid metabolism. Furthermore, Kang et al. (2021) further demonstrated that heat-killed *L. rhamnosus* reduced inflammatory markers such as TNF-α, TGF-β1, and cortisol. Thus, dietary supplementation with postbiotics from inactivated *Lactobacillus* may represent an effective strategy to mitigate weaning stress in calves by improving antioxidant capacity, modulating inflammation, and supporting growth.

Limosilactobacillus ingluviei (L. ingluviei), a newly identified probiotic species with growth-enhancement effects. Sirisopapong et al. (2023) documented that *L. ingluviei* displayed robust resistance against acidic conditions and bile salts, along with notable antibacterial properties, tolerance to antibiotics, and a strong affinity for intestinal epithelial cells. Murakami et al. (2024) reported that orally administered probiotic *L. ingluviei* C37 increased lysozyme levels and upregulated the expression of genes associated with tight junction proteins. Additionally, Angelakis et al. (2012) reported that feeding mice with *L. ingluviei* could increase weight gain, fecal *L*. spp. and *Firmicutes* DNA copy numbers. Mandal et al. (2013) demonstrated that *L. ingluviei* ADK10 exhibits potential free radical scavenging activity.

Transcriptome sequencing technology (RNA-seq) allows for the comprehensive and precise identification of transcripts in specific tissues, enabling the detection of subtle changes in gene expression (Kim et al., 2022). This facilitates transcriptomic analyses that further elucidate various biological processes, including metabolic pathways and signal transduction, thereby advancing our understanding of the molecular mechanisms underlying phenotypic variation (Zhao et al., 2020). While limited reports on postbiotics have primarily focused on cell-free supernatants, the functional potential of inactivated microbial cells in ruminant health remains largely unexplored. Therefore, this study aimed to investigate the postbiotic derived from heat killed *Limosilactobacillus ingluviei* C37 on growth performance, antioxidant capacity in calves during weaning period. In addition, transcriptome sequencing of the liver and

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jejunum was conducted to elucidate the molecular mechanisms by which postbiotics alleviate weaning stress.

1.2 Research objectives

1.2.1 To study the effect of postbiotic from heat killed *Limosilactobacillus ingluviei* C37 on growth performance and plasma antioxidant capacity in calves during weaning period.

1.2.2 To study the effect of postbiotic from heat killed *Limosilactobacillus ingluviei* C37 on liver transcriptome profile in calves during weaning period.

1.2.3 To study the effect of postbiotic from heat killed *Limosilactobacillus ingluviei* C37 on jejunal epithelium transcriptome profile in calves during weaning period.

1.3 Research hypotheses

1.3.1 Dietary supplementation with heat killed *Limosilactobacillus ingluviei* C37 can improve growth performance, enhance antioxidant capacity in calves.

1.3.2 Dietary supplementation with heat killed *Limosilactobacillus ingluviei* C37 can modulate liver transcriptomic profiles, by affecting genes related to antioxidant defense and lipid metabolism in calves.

1.3.3 Dietary supplementation with heat killed *Limosilactobacillus ingluviei* C37 can modulated jejunal transcriptomic profiles, by affecting genes related to immunity and absorption in calves.

1.4 Scope of the study

This study aimed to evaluate dietary heat killed *Limosilactobacillus ingluviei* C37 on growth performance, antioxidant capacity in calves during the weaning period. In addition, the study includes an analysis of liver and jejunal transcriptomic profiles to explore the molecular mechanisms underlying the observed physiological effects, particularly genes related to antioxidant defense, lipid metabolism, and immune regulation. The outcomes of this study will provide insight into the potential of heat killed *Limosilactobacillus ingluviei* C37 as a feed additive to enhance the health and productivity of calves during the weaning period.

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1.5 Expected benefits

1.5.1 Dietary with heat killed *Limosilactobacillus ingluviei* C37 can improve growth performance of calves during the weaning period, specifically by increasing body weight gain, and feed efficiency.

1.5.2 Dietary supplementation with heat killed *Limosilactobacillus ingluviei* C37 can alleviate the stress response in calves during the weaning period, specifically by enhancing antioxidant enzymes capacity, reductions of lipid peroxidation and inflammatory response. Additionally, it may to upregulate genes involved in lipid metabolism, fatty acid oxidation, and energy metabolism.

1.5.3 Dietary supplementation with heat killed *Limosilactobacillus ingluviei* C37 is expected to change transcriptomic profile in both liver and jejunum. Specifically, in the liver, metabolic regulatory pathways may be enhanced by heat killed *Limosilactobacillus ingluviei* C37 supplementation, supporting improved energy and lipid metabolism. In the jejunum, gene expression changes are anticipated to strengthen epithelial barrier function, enhance detoxification capacity, and modulate immune responses.

1.5.4 The findings of this study may expand our understanding of the application of postbiotics. It plays multifunctional roles in regulating metabolism and improving antioxidant capacity suggest promising prospects for extension to other young ruminants.

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CHAPTER II

LITERATURE REVIEWS

2.1 Calf weaning

Weaning stress mainly includes nutritional stress and psychological stress (Weary et al., 2008). Of which, nutritional stress is caused by the transformation of feed morphology and nutrient composition (Liu et al., 2023). The gastrointestinal system of calves is underdeveloped, the gastrointestinal microecosystem is incomplete, the immune and antioxidant capacity is low, and the digestive and absorption capacity is weak (Clark et al., 2017). Resulting in reduction of nutrient intake, produces stress and immune dysfunction (Dhabhar, 2002). In psychological stress, separation from the cow induces significant stress in calves, leading to negative emotions and a pessimistic judgment bias (Enríquez et al., 2010). Daros et al. (2014) found that separating dairy calves from their dams reduced “GO” responses to ambiguous cues (72% to 62%), similar to the negative emotional state caused by hot-iron dehorning. Therefore, weaning stress impairs calf health through a variety of pathways such as physiological, metabolic, and immune defense.

Weaning stress may disrupt gastrointestinal barrier function and increase intestinal permeability, ultimately resulting in diarrhea in calves (Wickramasinghe et al., 2022). The disruption of the intestinal barrier increases susceptibility to pathogens, especially considering the concurrent immaturity of the immune system. During weaning, the active immune system of the calf is still developing, while passive immunity from maternal milk has diminished (Nocek et al., 1984; Zhang et al., 2019). Weaning stress may lower antibody levels and weaken cellular immunity in calves, leading to a decline in their passive immunity (Liu et al., 2023). Bacterial challenges during this period may suppress immune function and trigger inflammatory responses (Zhang et al., 2019). Wang et al. (2022) reported that weaning reduces serum concentrations of immunoglobulins IgG and IgM in calves. In addition, weaning stress can alter cytokine secretion and upregulate the expression of related immune response

genes. For instance, O'Loughlin et al. (2014) observed elevated mRNA levels of IL-1β, IL-8, IFN-γ, and TNF-α in response to weaning stress. Additionally, weaning stress alters gut microbiota composition, which in turn influences intestinal and immune functions in calves. Moreover, earlier studies have shown that weaning disturbs the oxidative-antioxidative balance, resulting in oxidative stress that ultimately triggers enterocyte apoptosis and induces cell cycle arrest in the small intestinal epithelium (Zhu et al., 2012; Zhu et al., 2013).

Previous studies have identified gut microbiota as a key regulator of intestinal immune responses (Huang et al., 2005). It alleviates enteritis by promoting the differentiation of intestinal T cells (Bousbaine et al., 2022). It also helps maintain intestinal homeostasis by inhibiting NF-κB pathway activation, thereby reducing pro-inflammatory cytokine production. This process increases IL-10 and IgE secretion, which preserves the gut's mechanical barrier and prevents apoptosis in colonic epithelial cells (Chen et al., 2021). Furthermore, gut microbiota contributes to the balance between T and B cells, suppresses IL-17 and TNF-α secretion, enhances intestinal barrier function, and alleviates colitis in animal models (Moratalla et al., 2016a; Moratalla et al., 2016b). In addition, gut microbiota supports intestinal maturation and angiogenesis, enhances the mucosal barrier, and facilitates nutrient absorption (Jandhyala et al., 2015).

2.2 Postbiotic

For decades, probiotics have been a staple in animal feed as additives to alleviate oxidative stress and improve immunity. Yet, the widespread presence of antibiotic resistance genes in these strains, and the demonstrated ability for these genes to transfer between organisms (Marteau and Shanahan, 2003). Additionally, high probiotic concentrations may disrupt cytokine balance and cellular functions, potentially altering long-term immune responses in patients with immune disorders (de Simone, 2019). Casts a long shadow over their suitability for continued use as live bacteria in future nutritional strategies. A substantial body of scientific evidence supports the beneficial effects of postbiotics on animal health, demonstrating that cell viability is not essential for their positive impact (Zorzela et al., 2017). Therefore, postbiotics offer a compelling alternative to traditional probiotics, with advantages such as reduced risk of probiotic-

related sepsis and antibiotic resistance, along with a longer shelf life due to the absence of cold chain requirements for maintaining viability (Shripada et al., 2020). This literature review aimed to explore the composition, mechanism, and application of postbiotic in animal production.

2.2.1 A definitional debate

The definition of postbiotics has sparked widespread debate in the scientific community, driving further research in this field. In 2013, Tsilingiri and Rescigno (2013) defined that postbiotics are probiotic-produced, soluble factors that have been isolated and characterized and are sufficient to elicit the desired response. Particularly controversial was a 2019 review by the International Scientific Association for Probiotics and Prebiotics (ISAPP) on the definition and scope of postbiotics, that is, “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host”. The resulting report was published by Salminen et al. (2021a). In contrast, Aguilar-Toalá et al. (2021) argue that the term "postbiotic" had been sufficiently defined in 2013 (Tsilingiri and Rescigno, 2013), and that the ISAPP definition (Salminen et al., 2021a) only introduced confusion rather than clarity to the field. Subsequently, Salminen et al. (2021b) reply to this, and indicating that consensus around a single definition had not been achieved by the scientific community. The ISAPP panel had several concerns with the 2013 definition of Tsilingiri and Rescigno (2013): “it requires a probiotic as a progenitor, creating the untenable situation in which a metabolite produced by an established probiotic is considered a postbiotic whereas the same metabolite produced by a non-probiotic microorganism is not; it does not distinguish between a product administered to a host and microbial metabolites that could be produced in situ; and it uses the phrase “beneficial effects to the host in a direct or indirect way” and does not specify a health benefit, leaving the door open to further debate on what types of benefit could be encompassed”.

This study does not discuss which definition is more accurate, for the convenience of the following statement, cell structural components and secreted metabolites are collectively referred to as postbiotics. This study used *Lactobacillus* as an example, which is the most commonly probiotic.

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2.2.2 Composition and function of postbiotic

Postbiotic contains a variety of bioactive substances, including peptidoglycans, surface proteins, cell wall polysaccharides, teichoic acids, secreted proteins and peptides, bacteriocins, organic acids, lipoteichoic acid (LTA), and short-chain fatty acids (Vinolo et al., 2011; Sun et al., 2018; Teame et al., 2020), which confer its antimicrobial activity and immunomodulatory ability. The cell surface components of *Lactobacillus* are considered key effector molecules, as they are the first part of the microbial cell to interact with host cells (Teame et al., 2020) (**Figure 2.1**).

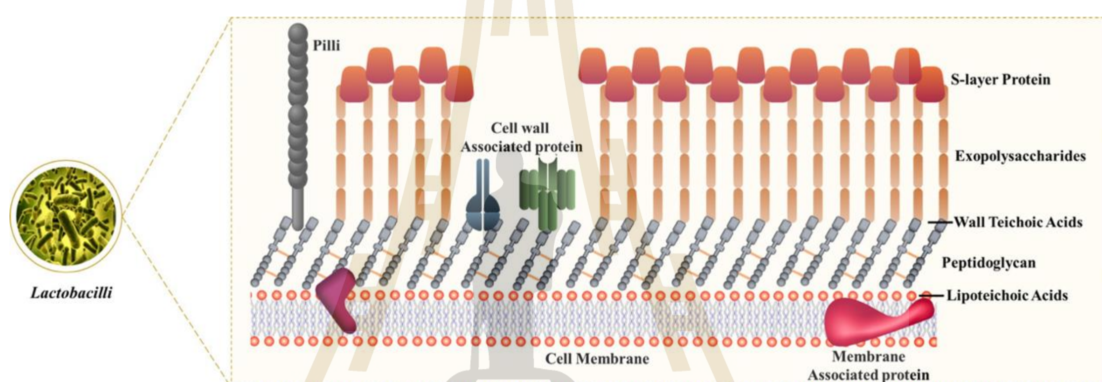

Figure 2.1 Cell wall of *Lactobacillus* (source: (Kumar et al., 2023)).

Peptidoglycan is a major component of the cell wall of *Lactobacillus* (Lebeer et al., 2010). During peptidoglycan synthesis and assembly, structural modifications can occur that increase bacterial susceptibility to autolysis, alter cell envelope hydrophobicity, and enhance lysozyme resistance (Yadav et al., 2018). Shida et al. (2009) reported that peptidoglycan of *Lactobacillus* inhibits interleukin-12 (IL-12) production through Toll-like receptor 2 (TLR2), which has been implicated in autoimmune and inflammatory bowel diseases. Similarly, Fernandez et al. (2011) reported that *Lactobacillus salivarius* Ls33 peptidoglycan activated dendritic cells and regulatory T-cell functions through recognition by nucleotide-binding oligomerization domain protein 2 (NOD2), effectively protecting mice from trinitrobenzene sulfonic acid (TNBS)-induced colitis.

Teichoic acid (TAs) is the second major component of the *Lactobacillus* cell wall (Kleerebezem et al., 2010), which can bind to the cytoplasmic membrane by

lipid anchors to form lipoteichoic acid (LTA) (Weidenmaier and Peschel, 2008). Previous studies reported that LTA of *Lactobacillus* can regulate immunity. For example, Noh et al. (2015) found that *L. plantarum* LTA attenuated the expression of IL-8 induced by Pam2CSK and exerted anti-inflammatory effects on human intestinal epithelial cells. Similarly, Kim et al. (2017) reported that LTA of *L. plantarum* also showed anti-inflammatory responses in porcine intestinal epithelial cells.

Exopolysaccharides (EPS) mediate adhesion, protect against pathogens, regulates intestinal permeability, and serve as a protective barrier (Castro-Bravo et al., 2018; Kim et al., 2018). Briefly, EPS exerts an inhibitory effect on bacterial adhesion to mucus through a competitive exclusion mechanism, occupying binding sites and thereby preventing pathogen attachment (López et al., 2012). Kšonžeková et al. (2016) demonstrated that EPS could inhibit the adhesion of enterotoxigenic Escherichia coli (ETEC) to IPEC-1 cells, thereby reducing the expression of proinflammatory cytokines such as IL-1β and IL-6. EPS was shown to suppress antigen-specific activation of $CD4^+$ T cells mediated by dendritic cells (DCs) (Yang et al., 2023). Ren et al. (2020) reported that EPS derived from L. casei ATCC 393 enhanced intestinal mucosal immunity by promoting the differentiation of $CD4^+$ T cells in Peyer's patches into T-helper 17 (Th17) cells. Additionally, EPS enhanced intestinal epithelial barrier function by activating signal transducer and activator of transcription 3 (STAT3), which upregulated the expression of tight junction proteins, including zonula occludin protein-1 (ZO-1) and occludin (Yang et al., 2023). Similarly, Liu et al. (2021) found that EPS of *L. helveticus* KLDS1.8701 upregulated the mRNA expression of claudins, occludin, ZO-1, and mucin proteins in mice, thereby enhancing intestinal barrier function. Additionally, EPS of the *Lactobacillus* exhibits potential antioxidant properties (Liu et al., 2011). Li et al. (2014) demonstrated that *Lactobacillus* EPS exhibited strong antioxidant activity, effectively scavenging DPPH, hydroxyl, and superoxide radicals, as well as inhibiting lipid peroxidation. Similarly, Li et al. (2021) found that EPS from *Lactobacillus rhamnosus* GG could mitigate H_2O_2-induced oxidative damage in intestinal porcine epithelial (IPEC-J2) cells. Furthermore, EPS can also regulate the energy metabolism of host. Zhang et al. (2017) found that the EPS isolated from *L. rhamnosus* GG inhibited adipogenesis, and deceased the level of triacylglycerols and cholesterol ester in the liver and serum in mice. Zhang et al. (2016) found that exopolysaccharides (EPS) from

Lactobacillus rhamnosus GG significantly decreased the triacylglycerol (TAG) accumulation without any inflammation, while EPS downregulated inflammation in adipose tissue and liver. Additionally, in high-fat diet mice, EPS administration lowered TAG and cholesterol ester levels in the liver and reduced serum TAG (Zhang et al., 2016).

S-layer proteins (SLP) of *Lactobacillus* play a critical role in host defense by competitively binding to intestinal epithelial cells, thereby inhibiting pathogen adhesion and infection (Chen et al., 2007; Johnson-Henry et al., 2007; Teame et al., 2020). Beyond their barrier function, these proteins also modulate the host immune response. For example, (Malamud et al., 2018) demonstrated that SLP of *Lactobacillus* can enhance macrophage responsiveness to lipopolysaccharides (LPS). Meng et al. (2014) reported that SLP of *Lactobacillus* effectively inhibited both the adhesion and invasion of Escherichia coli ATCC 43893 in HT-29 intestinal epithelial cells.

On the other hand, metabolites of *Lactobacillus* including short chain fatty acids (SCFAs), lactic, and bacteriocins also show probiotic effects (Thanh et al., 2009). Among these, short-chain fatty acids (SCFAs) and lactic contribute to a reduction in cecal pH, which suppresses the growth of pH-sensitive pathogens (Fernández-Rubio et al., 2009). SCFAs can also infiltrate microbial cells, disturbing ion equilibrium by altering intracellular charge distribution, thereby disrupting cellular function and inhibiting pathogen development (Deleu et al., 2021). Beyond antimicrobial effects, these metabolites are involved in metabolic regulation. Specifically, SCFAs such as butyric and propionic acids activate PPARγ (Alex et al., 2013), a key transcription factor involved in lipid homeostasis. This activation enhances energy utilization (Gao et al., 2009), leading to reductions in body weight and hepatic triglyceride accumulation (den Besten et al., 2015). Additionally, SCFAs (e.g. propionic acid) contributes to cholesterol reduction Bosch et al. (2014). This effect is achieved by decreasing systemic lipid levels through the inhibition of hepatobiliary sterol synthesis and/or promoting cholesterol redistribution from plasma to the liver (Pereira and Gibson, 2002).

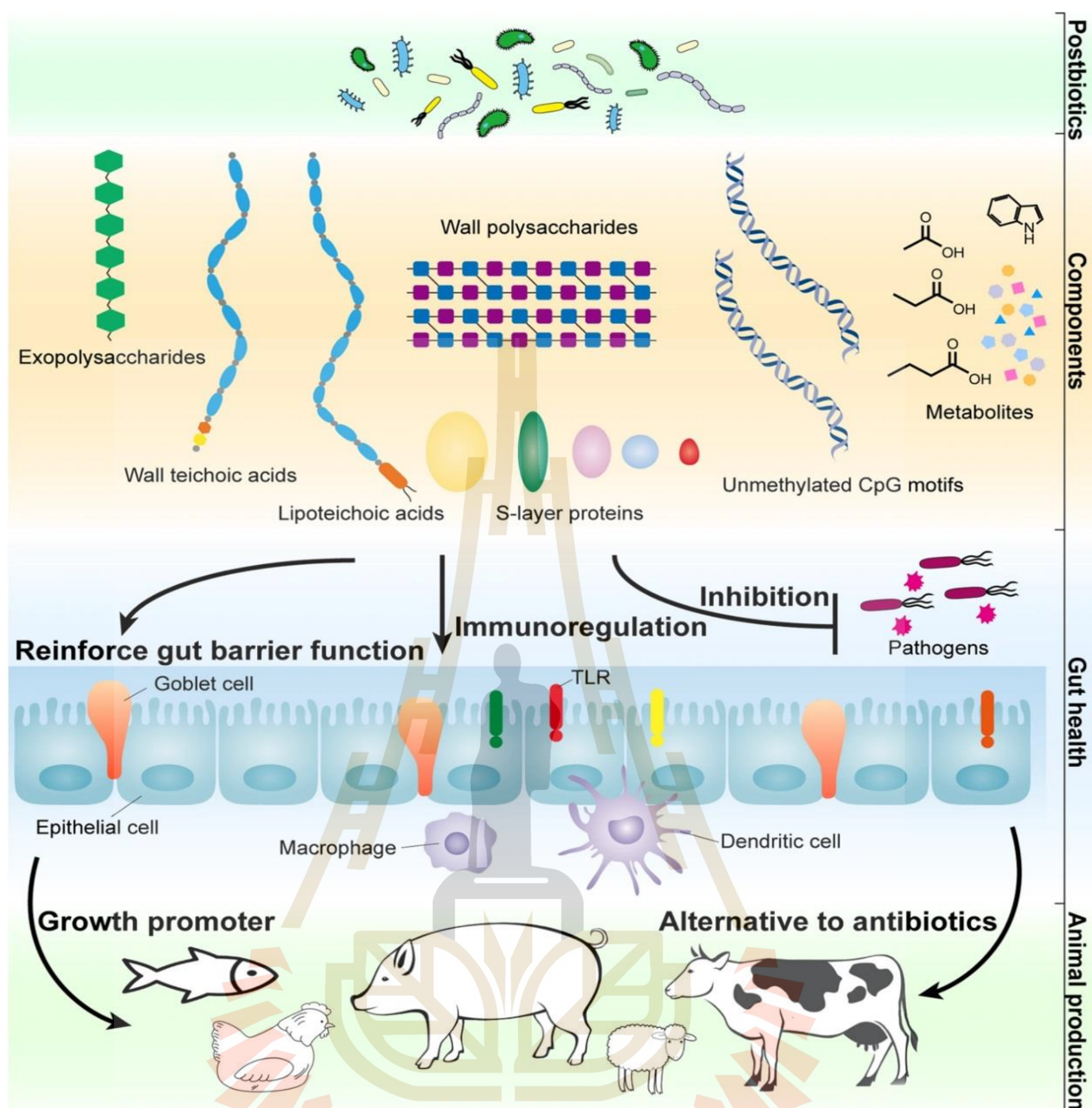

Figure 2.2 Mechanism of *Lactobacillus* on intestine in livestock (source: (Zhong et al., 2022)).

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2.2.3 Postbiotic in animal nutrition

Postbiotics have been extensively studied for their role in improving animal production performance and enhancing feed efficiency. For example, Fang et al. (2024) found that feeding broilers with different levels of commercial postbiotic from dead cell of Bacillus subtilis ACCC 11025 showed an increased body weight gain (BWG), and feed efficiency. Similarly, Chang et al. (2022) reported that supplementation postbiotic metabolites from *L. plantarum* RS5 decreased feed conversion ratio (FCR) in broiler. Additionally, similar benefits have been observed in ruminants. For example,

Izuddin et al. (2019b) reported that feeding post-weaning lambs with postbiotic metabolites from *L. plantarum* RG14 increased BWG and decreased FCR, while improving nutrient digestibility (e.g. CP, NDF). In contrast, Fernández et al. (2023) reported that feeding goats with postbiotic from yeast fermentation product had not affect dry matter intake (DMI), and nutrient digestibility. On the other hand, Ríus et al. (2022) found that feeding calves with postbiotic from *Aspergillus oryzae* decreased feed intake (FI), but no affect BWG and feed efficiency (FE). Furthermore, Vicente et al. (2024) demonstrated that feeding cows with 8 g/d commercial postbiotic (Pentabiol SL) increased DMI, nutrient digestibility (e.g. DMI, DNDF) (**Table 2.1**).

2.2.4 Effect of postbiotic on blood metabolite and antioxidant capacity

Loh et al. (2014) investigated the effect of feeding a postbiotic mixture to laying hens on CHOL, which was reduced in the blood of single machines after feeding postbiotic compared with the control. Izuddin et al. (2019a) reported that feeding post-weaning lambs with postbiotic metabolites from *L. plantarum RG14* increased blood total protein (TP), blood urea nitrogen (BUN), and globulin levels, without affecting triglyceride (TRIG), and cholesterol (CHOL). Dai et al. (2024) observed that feeding transition dairy cows with postbiotic from *Saccharomyces cerevisiae* had no affect blood metabolic parameters. Similarly, similar results were observed in piglets fed with postbiotic from *Lactobacillus reuteri* (Sun et al., 2025). Monika et al. (2024) reported that feeding broiler chicken with different levels of postbiotic metabolites from *Lactobacillus acidophilus* decreased CHOL without affect TRIG levels (**Table 2.2**).

Chang et al. (2022) found that feeding broiler with postbiotic metabolites from *L. plantarum RG11* showed an increase total antioxidant capacity (TAOC), and no affect glutathione peroxidase (GPX), catalase (CAT), and superoxide dismutase (SOD), additionally, *L. plantarum RI11* increased TAOC and CAT levels. Similarly, Humam et al. (2020) reported that dietary *L. plantarum RI11* had increased TAOC and CAT levels in broiler. In contrast, Xu et al. (2023) found that feeding lactating sows with yeast-derived postbiotic did not affect antioxidant capacity including TAOC, GPX, SOD, and malondialdehyde (MDA). On the other hand, Izuddin et al. (2020) observed that feeding post-weaning lambs with postbiotic metabolites from *L. plantarum RG14* increased GPX, and decreased MDA levels. Similarly, Dai et al. (2024) reported that feeding transition dairy cows with postbiotic from *Saccharomyces cerevisiae* reduced MDA levels (**Table 2.3**).

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Table 2.1 Effect of dietary postbiotic on growth performance in animals.

References	Species	Postbiotic	Type[1]	Levels	Performance[2]
Fang et al. (2024)	Broiler	Bacillus subtilis ACCC 11025	Dead cell	0.015%	BWG (↑); FI (-); FE (↑); ALB (-); GLB (-); TP (-)
				0.030%	BWG (↑); FI (-); FE (↑); ALB (-); GLB (-); TP (↑)
				0.045%	BWG (↑); FI (-); FE (↑); ALB (↑); GLB (-); TP (-)
Chang et al. (2022)	Broiler	L. plantarum RS5	CFS; v/w	0.01%	FI (-); BWG (-); FCR (↓)
Izuddin et al. (2019b)	Lambs	*L. plantarum RG14*	CFS; v/w	0.9%	BWG (↑); FI (-); FCR (↓); DDM (↑); DEE (-); DCP (↑); DNDF (↑)
Ríus et al. (2022)	Calves	*Aspergillus oryzae*	-	3 g	BWG (-); FI (↓); FE (-)
Vicente et al. (2024)	Cows	Pentabiol SL	-	8 g/d	DMI (↑); DDM (↑); DCP (-); DNDF (↑)
Fernández et al. (2023)	Goats	yeast fermentation product	CFS	3.75 g/d	DMI (-); DDM (-); DCP (-)

[1] CFS, cell-free supernatant; v/w, the ratio of volume to weight.

[2] BWG, body weight gain; FI, feed intake; FE, feed efficiency; FCR, feed conversion ratio; ALB, albumin; GLB, globulin; TP, total protein; DDM, digestibility of dry matter; DEE, digestibility of ether extract; DCP, digestibility of crude protein; DMI, dry matter intake; DNDF, digestibility of neutral detergent fiber.

[3] (↑), the data was significantly increased by postbiotic supplementation; (↓) the data was significantly decreased by postbiotic supplementation; (-), no significant difference was observed. The same below.

Table 2.2 Effect of dietary postbiotic on blood metabolic parameters.

References	Species	Postbiotic	Type[1]	Levels	Performance[2]
Izuddin et al. (2019a)	Lambs	*L. plantarum RG14*	CFS; v/w 10^9 CFU/mL	0.9%	TP (↑); BUN (↑); GLU (↑); TRIG (-); CHOL (-)
Sun et al. (2025)	Piglets	Lactobacillus reuteri		500 mg/kg	TP (-); ALB (-); GLB (-); TRIG (-); CHOL (-)
Dai et al. (2024)	Dairy cows	*Saccharomyces cerevisiae*		19 g/d	TP (-); ALB (-); GLB (-); CHOL (-); GLU (-); BUN (-); AST (-)
		RGS5 + RI11 + RG14	10^9 CFU/mL	0.6%	CHOL (↓);
Loh et al. (2014)	Laying hens	RI11 + RG14 + RG11	10^9 CFU/mL	0.6%	CHOL (↓);
		RI11 + TL1 + RG11	10^9 CFU/mL	0.6%	CHOL (↓);
				0.2%	CHOL (↓); TRIG (-)
Monika et al. (2024)	broiler	*Lactobacillus acidophilus*	CFS; (v/w)	0.4%	CHOL (↓); TRIG (-)
				0.6%	CHOL (↓); TRIG (-)

[1] ALB, albumin; GLB, globulin; TP, total protein; BUN, blood urea nitrogen; GLU, blood glucose; TRIG, triglycerides; CHOL, cholesterol; AST, aspartate aminotransferase.

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Table 2.3 Effect of dietary postbiotic on blood antioxidant capacity.

References	Species	Postbiotic	Type	Levels	Performance[1]
Chang et al. (2022)	Broilers	*L. plantarum RG11*	CFS; v/w	0.01%	TAOC (↑); GPX (-); CAT (-); SOD (-)
		L. plantarum RI11	10^9 CFU/mL		TAOC (↑); GPX (-); CAT (↑)
Dai et al. (2024)	Transition dairy cows	*Saccharomyces cerevisiae*		19 g/d	TAOC (-); GPX (-); MDA (↓)
Humam et al. (2020)	Broilers	*L. plantarum RI11*	CFS; v/w 10^9 CFU/mL	0.3%	TAOC (↑); GPX (-); CAT (↑); SOD (-)
Xu et al. (2023)	Lactating sows	Yeast-derived		2 g/kg	TAOC (-); GPX (-); SOD (-); MDA (-)
Izuddin et al. (2020)	Post-Weaning Lambs	*L. plantarum RG14*	CFS; v/w; 10^9 CFU/mL	0.9%	SOD (-); GPX (↑); MDA (↓)

1 TAOC, total antioxidant capacity; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde.

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CHAPTER III
EFFECT OF DIETARY POSTBIOTIC LIC37 ON GROWTH PERFORMANCE AND HEMATOLOGICAL PARAMETERS IN CALVES DURING WEANING PERIOD

3.1 Abstract

This study aimed to estimate postbiotic from heat killed *Limosilactobacillus ingluviei* C37 (postbiotic LIC37) on growth performance, antioxidant capacity, and ruminal bacteria community in calves during weaning period. A total of 14 Holstein bull calves (age: 5.71 ± 1.14 days) were blocked by body weight (BW) (37.34 ± 3.19 kg, and 28.83 ± 2.92 kg; mean ± SD) and randomly allocated into two groups (n = 7 per group): a control group receiving a basal diet (CON), and a treatment group feeing with 1 g/d of postbiotic LIC37 (10^8 CFU/g) (TRT group). Calves received milk replacer (MR) at 1.75% of BW (based on air-dry weight) and the amount was adjusted weekly according to body weight, while fresh and clean water was provided *ad libitum*. All calves had free access to starter starting on day 33. On day 82, the MR solution was reduced to 50% of the previous week's amount, and calves were completely weaned by day 89. Blood samples were collected on day 73 (pre-weaning), day 83 (mid-weaning), and day 90 (post-weaning). The results showed that postbiotic supplementation improved feed efficiency at day 32-89, and whole stage ($P < 0.05$). Compared to the CON group, postbiotic reduced globulin, total protein, Neutrophil (Neu) and the ratio of neutrophil to lymphocyte (NLR) levels in the TRT group ($P < 0.05$). Additionally, the serum urea nitrogen (BUN), triglyceride (TRIG), and cholesterol (CHOL) levels tended to decrease ($P < 0.1$) with postbiotic supplementation. Feeding postbiotic led to a reduction ($P < 0.05$) in cortisol and malondialdehyde (MDA), and increasing catalase (CAT) levels ($P < 0.05$), while there was a tendency for increased for glutathione peroxidase (GPX) level and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging capacity ($P < 0.1$).

Overall, calves fed postbiotic LIC37 exhibited improved feed efficiency, modulated serum biochemical and enhanced antioxidant capacity in calves, indicating its potential as a functional nutritional strategy to support calf health and development during weaning.

Keywords: Bacteria; Calf weaning; *Limosilactobacillus ingluviei*; Postbiotic.

3.2 Introduction

Weaning is a pivotal challenge experience in early calf development, as it transitions from a pseudo-monogastric to a functioning ruminant (Welk et al., 2024), and is often accompanied by stress that triggers an increase in reactive oxygen species (ROS), posing a challenge for antioxidant defenses in calves (Eitam et al., 2010). Weaning is frequently associated with a decline in average daily gain (ADG), and impaired rumen development and function (Strzetelski et al., 2001; Hill et al., 2006), or decreased digestibility of the starter feed (Terré et al., 2007). Therefore, mitigating the oxidative stress of calves during weaning period is an urgent issue to be addressed.

Probiotic can potentially improve growth performance (Wang et al., 2023), modulate gastrointestinal microbiota, and enhance ruminant health (Markowiak and Śliżewska, 2018)For example, Frizzo et al.(2010)observed that dietary supplementation with lactic acid bacteria increased average live weight gain and feed intake of calves. Additionally, previous study demonstrated that feed dairy calves with postbiotic increased antioxidant enzymes concentration such as GSH, reduced MDA levels (Yao et al., 2020).

However, use of live cells of probiotic in individuals with weakened immune systems, increased inflammatory responses, and/or compromised mucosal barrier function may convert harmless probiotics that are "generally considered safe" into harmful microorganisms (Besselink et al., 2008). Previous studies showed that feeding with live cell *Lactobacillus* may cause serious infections (e.g., sepsis, pneumonia, and meningitis) in immunocompromised newborns (Dani et al., 2016). On the other hand, an inherent challenge with probiotics lies in their necessity to effectively colonize the gastrointestinal tract and maintain viable populations (Adams, 2010). Achieving this

requires probiotics to be of a specific strain tailored to the host animal, presenting a practical difficulty as standardized products necessitate commercial production (Adams, 2010).

Postbiotics, the inactivated forms of probiotics that replicate their beneficial effects, present a safer alternative for application, offering similar benefits without the complexities of live cultures (Wagner et al., 2000; Gill and Guarner, 2004). Despite numerous reports on the use of postbiotic to improve growth performance and reduce weaning stress in ruminants (Nagashima et al., 2010; Ikehata et al., 2023). However, the application of postbiotic from heat killed *Limosilactobacillus ingluviei* C37 in ruminants is still limited.

3.3 Objective

This study was to estimate the effect of dietary postbiotic LIC37 supplementation on growth performance, serum biochemical parameters, and plasma antioxidant capacity in calves during the weaning period.

3.4 Materials and methods

3.4.1 Ethics statement

The feeding trial took place at the Suranaree University of Technology (SUT) farm (Nakhon Ratchasima, Thailand). All animal procedures were performed in accordance with the relevant guidelines and regulations, and were approved following the ethics by the Animal Ethics Committee of Suranaree University of Technology issued a statement approving the experimental protocol (SUT-IACUC-0020/2023).

3.4.2 Animals, treatment and sampling method

The *Limosilactobacillus ingluviei* (*L. ingluviei*) C37 strain was obtained from the Laboratory of Monogastric Animal Nutrition and Feed Science at Suranaree University of Technology (SUT). The isolation and cultivation of *L. ingluviei* C37 were thoroughly detailed by Sirisopapong et al. (2023). The postbiotic LIC37 was achieved by heat-killing the cells at 80°C for 30 minutes, following the method described by Tsukagoshi et al. (2020).

Fourteen Holstein bull calves were obtained from a local dairy farm at the age of 5.71 ± 1.14 d. Each calf received 2 liters of colostrum within 3 hours of birth, followed by an additional 2 liters within the next 12 hours. All calves with serum total protein levels exceeding 5.6 g/dL at 24 hours of birth were selected, confirming the successful transfer of passive immunity (Hernandez et al., 2016). The calves were gradually transitioned from colostrum to bucket-fed milk replacer (MR) beginning at three days of age before being transferred to the SUT farm. Upon arrival, they received an immediate intramuscular injection of vitamin B12 Catosal™, OLIC Co. Ltd, Ayutthaya, Thailand) at a 5% (mL/kg) dosage. According to previous study of Domple et al. (2020), a sample size of 7 was determined to provide power greater than 0.8 and a significance level of 0.05 for detecting a 2.72 nmol/L difference in serum cortisol. Thus, fourteen Holstein bull calves were blocked into 2 groups by body weight (37.34 ± 3.19 kg, and 28.83 ± 2.92 kg; mean ± SD), and randomly assigned to 2 treatments (7 per treatment). Namely, CON group (without postbiotic) and TRT group with 1 g/d of postbiotic LIC37 (10^8 CFU/g). The dosage was determined based on conversions from previous studies (Thorsteinsson et al., 2020; McNeil et al., 2024). Each calf was individually housed in a 2.2 m × 2.4 m pen equipped with rubber mats and wood pellets. The soiled wood pellets were removed daily, and fresh wood pellet was provided weekly.

Commercial MR was procured from Dairy-Rich Co. Ltd (Bangkok, Thailand), with its nutrient composition detailed in **Table 3.1**. The MR for calves was provided twice daily at 08:00 and 16:00 at a concentration of 15%, equivalent to 1.75% of BW (base on air-dry). The feeding amount was adjusted weekly based on BW. The postbiotic LIC37 was mixed into the morning MR feeding, while fresh and clean water was available *ad libitum*. As the sole solid feed, the commercial starter (Charoen Pokphand Foods, Bangkok, Thailand) was provided *ad libitum* from day 33 of the experiment.

Table 3.1 Chemical composition of milk replacer and commercial calf starter.

Item[1]	MR	Starter
DM, %	97.40	90.94
Ash, % DM	8.87	8.97
CP, %DM	22.67	23.79
Fat, %DM	17.74	3.99
NDF, %DM	-	52.09
ADF, %DM	-	12.24

[1] MR, milk replacer; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber.

The 90-day feeding trial began on the day the calves arrived at SUT farm. The weaning process was completed within one week. Specifically, it started on day 82, with the MR solution reduced to 50% of the previous week's amount. Calves were completely weaned by day 89. This feeding strategy was intended to induce weaning stress (Van Niekerk et al., 2021).

MR and starter samples were gathered every two weeks and combined into a composite sample. These samples were then stored at -20°C for later analysis. On day 76 (pre-weaning), 83 (mid-weaning), and 90 (post-weaning), prior to the morning feeding. Approximately 8 mL of blood samples were collected by jugular venipuncture using K_3EDTA vacuum tube, and a sterile tube without anticoagulation. A part of the blood samples was transferred to the SUT hospital for detected serum biochemical parameters including glucose (GLU), blood urea nitrogen (BUN), Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Triglyceride (TRIG), total Cholesterol (CHOL), albumin, globulin, total protein, and complete blood count (CBC) parameters. The other part of the blood samples was centrifuged at 3,000 × g for 10 min at room temperature. The plasma and serum were harvested and stored at -80°C for future analysis of antioxidant capacity and cortisol concentration.

3.4.3 Chemical analysis

The dry matter (DM), crude protein (CP), ash, and fat contents were analyzed following the methods of the Association of Official Analytical Chemists (Helrich, 1990). DM (AOAC 930.15) content was measured using an oven at 105 °C for

24 hours; CP (AOAC 984.13) content was determined using the Kjeldahl system (N × 6.25); ash (AOAC 942.05) content was assessed through incineration at 550°C for 4 hours in a Carbolite AAF1100 Ashing Furnaces (Germany); fat (AOAC 920.39) content was analyzed using the Soxtec system (Foss Co., Ltd, Hillerød, Denmark). The ADF and NDF contents were determined according to the method of Van Soest et al. (1991).

The plasma antioxidant capacity including total antioxidant capacity (TAOC), superoxide dismutase (SOD), glutathione peroxidase (GPX), malondialdehyde (MDA), catalase (CAT), and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) were used commercially available kits: E-BC-K136-M (Elabscience, Houston, USA), KTB1030 (Abbkine, Wuhan, China), KTB1640 (Abbkine, Wuhan, China), KTB1050-EN (Abbkine, Wuhan, China), KTB1040 (Abbkine, Wuhan, China), and E-BC-K807-M (Elabscience, Houston, USA) according to the manufacturer's instructions. The concentration of serum cortisol was determined by ELISA assay kit (Ann Arbor, Michigan, USA) according to the manufacturer's instructions.

3.4.4 Data and statistical analysis

The average daily gain (ADG) was calculated by subtracting the initial BW from the final BW and dividing the result by the number of trial days. Feed efficiency was determined as the ratio of ADG (or BW gain) to total dry matter intake (TDMI) according to method of McCoard et al. (2019).

Normally of data distribution was confirmed using the Shapiro-Wilk procedure of SPSS (version 27, Chicago, IL, USA). Non-normally distributed data were transformed using natural logarithms or raising variables to the power of lambda. The TRANSREG procedure in SPSS is used for Box-Cox analysis to calculate the lambda value. Data subject to transformation were used to calculate P values. Levene's test was used to assess the homogeneity of variances. If the test indicated non-homogeneous variances ($P < 0.05$), the Kruskal-Wallis test was applied for group comparisons. Subsequently, the blood parameters including GLU, BUN, AST, ALT, ALP, TRIG, CHOL, albumin, globulin, AGR, total protein, CBC parameters, cortisol, TAOC, SOD, GPX, CAT, MDA, and DPPH scavenging capacity were analyzed as a complete randomized block design using the General Linear Model (GLM) in SPSS to analyze variables that were repeatedly measured over time. The growth performance parameters including ADG, MDMI, SDMI, TDMI, and feed efficiency, were analyzed

without being repeatedly measured. The model included the fixed effects of time, block, treatment, and time × treatment interaction, and sampling time was the repeated measure, the calf within treatment as a random effect. Sphericity was assessed using Mauchly's test, with the Greenhouse-Geisser correction applied whenever the assumption was breached. The significance level was set a P < 0.05, while trends were considered when P ≤ 0.10.

3.5 Results

3.5.1 Effect of postbiotic LIC37 on growth performance

As shown in **Table 3.2**, the postbiotic LIC37 supplementation did not affect BW change, including final BW, ADG, and BW gain (P > 0.05). Similarly, no differences were observed in feed intake, whether MDMI, SDMI, or TDMI (P > 0.05). Notably, the TRT group showed improved feed efficiency compared to CON group during day 33-90 (P = 0.014), and the overall period (day 1-89) (P = 0.035), while no significant was observed during the initial period (days 1-32) (P > 0.05).

3.5.2 Effect of postbiotic LIC37 on blood biochemical parameters

As shown in **Table 3.3,** postbiotic LIC37 supplementation decreased globulin and total protein levels (P < 0.05) without affect glucose, ALT, AST, ALT/AST, and albumin levels (P > 0.05). Additionally, postbiotic LIC37 supplementation significantly increased AGR levels (P = 0.027). However, there was a tendency for decreased concentrations of BUN (P = 0.052), CHOL (P = 0.092), and TRIG (P = 0.072) with postbiotic LIC37 supplementation. Moreover, weaning had a significantly effect on BUN, ALT, AST, TRIG, CHOL, globulin, and total protein levels (P < 0.05), while GLU and ALT/AST levels remained unaffected (P > 0.05). There was also a tendency for decreased concentrations of albumin (P = 0.078) and AGR (P = 0.091) levels. Furthermore, a significant interaction effect between group and time was observed for ALT, TRIG, and CHOL (P < 0.05).

Table 3.2 Effect of postbiotic derived from heat killed *Limosilactobacillus ingluviei* C37 supplementation on growth performance in calves during 1-89 days of age.

tem[1]	CON	TRT	SEM	P value
nitial BW, kg	33.64	33.74	2.076	0.985
Final BW, kg	68.20	78.44	6.886	0.198
Day 1-32				
ADG, g/d	232.14	234.55	43.723	0.887
MDMI, g/d	692.69	678.83	23.228	0.420
Feed efficiency	0.33	0.34	0.059	0.538
Day 33-89				
ADG, g/d	475.94	652.41	86.149	0.179
MDMI, g/d	791.64	827.82	65.483	0.597
SDMI, g/d	317.65	412.83	89.331	0.338
TDMI	1109.29	1240.64	138.093	0.460
Feed efficiency	0.40	0.53	0.032	0.014
Day 1-89				
BW gain, kg	34.56	44.69	5.707	0.139
MDMI, kg	66.50	68.08	4.224	0.757
SDMI, kg	17.79	23.12	5.003	0.338
TDMI, kg	84.29	91.20	8.055	0.498
Feed efficiency	0.39	0.49	0.033	0.035

[1]CON, control group (base diet); TRT, base diet with 1g of postbiotic (10^8 CFU/g of inactivated *L. ingluviei* C37); SEM, standard error of the mean; BW, body weight; ADG, average daily gain; MDMI, milk replacer intake based on dry matter; SDMI, starter intake base on dry matter; TDMI, total dry matter intake.

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Table 3.3 Effect of postbiotic derived from heat killed *Limosilactobacillus ingluviei* C37 supplementation on serum biochemical parameters in calves during 1-89 days of age.

	Time[1]							P value[2]		
	Day 76		Day 83		Day 89					
Item[3]	CON	TRT	CON	TRT	CON	TRT	SEM	Group	Time	GxT
Albumin, g/dL	2.79	2.87	2.91	2.97	3.14	3.34	0.069	0.433	0.078	0.299
Globulin, g/dL	3.31	2.76	3.61	3.01	3.87	3.37	0.087	0.008	0.006	0.596
AGR	0.85	1.04	0.81	0.99	0.82	1.01	0.029	0.027	0.091	0.921
Total protein, g/dL	6.10	5.53	6.53	5.63	7.01	6.71	0.117	0.027	0.002	0.252
GLU, mg/dL	73.00	75.86	69.57	71.00	65.29	68.14	2.329	0.673	0.506	0.746
BUN, mg/dL	8.76	7.44	9.66	8.89	16.64	10.11	0.280	0.052	<0.001	0.248
CHOL, mg/dL	122.00	116.57	110.86	97.43	100.71	85.29	3.514	0.092	<0.001	0.016
TRIG, mg/dL	29.86	28.14	23.43	20.14	16.86	12.29	1.470	0.072	<0.001	0.020
ALT, U/L	5.71	5.86	6.71	6.14	9.14	8.43	0.346	0.397	0.008	0.040
AST, U/L	51.29	50.00	52.29	51.00	56.71	54.29	0.738	0.234	0.002	0.549
ALT/AST, %	11.15	11.70	12.81	12.06	16.09	15.58	0.528	0.721	0.309	0.209

[1] Meaning sampling time.

[2] GxT, the interaction between group and time.

[3] AGR, the ratio of albumin to globulin; GLU, glucose; BUN, blood urine nitrogen; TRIG, Triglycerides; CHOL, Cholesterol; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALT/AST, the ratio of alanine aminotransferase to aspartate aminotransferase.

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3.5.3 Effect of Postbiotic LIC37 supplementation on complete blood count in calves

As shown in **Table 3.4**, postbiotic LIC37 supplementation did not affect RBC, HGB, and HCT levels ($P > 0.05$). The weaning had no effect on most parameters ($P > 0.05$), but it significantly affected HGB level ($P = 0.036$). Compared with the CON group, postbiotic LIC37 supplementation significantly decreased the levels of WBC, Neu, and NLR ($P < 0.05$), whereas Lymp level remained unaffected ($P = 0.126$). Furthermore, neither weaning nor the interaction between treatment and weaning had any significant effect ($P > 0.05$) on WBC, Neu, Lymp, or NLR levels, respectively.

3.5.4 Effect of postbiotic LIC37 on serum cortisol and plasma antioxidant capacity

As shown in **Table 3.5,** The postbiotic LIC37 supplementation significantly decreased cortisol and MDA levels ($P < 0.05$) while increasing CAT levels ($P = 0.002$). Additionally, postbiotic supplementation showed a tendency for increased GPX level ($P = 0.085$) and DPPH scavenging capacity ($P = 0.060$). On the other hand, the weaning had significantly affected the concentrations of cortisol, SOD, GPX, CAT, MDA, and DPPH scavenging capacity ($P < 0.05$), whereas TAOC remained unaffected ($P > 0.05$). Moreover, a significant interaction effect between group and time was observed for cortisol, GPX, and MDA levels ($P < 0.05$).

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Table 3.4 Effect of postbiotic derived from heat killed *Limosilactobacillus ingluviei* C37 supplementation on complete blood count in calves during 1-89 days of age.

	Time									
	Day 76		Day 83		Day 89			P value		
Item[1]	CON	TRT	CON	TRT	CON	TRT	SEM	Group	Time	GxT
RBC, $\times 10^6$ cells/mm^3	8.13	8.19	8.64	8.26	8.43	8.21	0.222	0.723	0.563	0.213
HGB, g/dL	7.77	7.89	9.07	9.26	8.74	8.90	0.230	0.740	0.036	0.930
HCT, %	24.43	25.71	27.43	28.57	27.57	27.36	0.612	0.555	0.137	0.248
WBC, $\times 10^3$ cells/mm^3	10.23	7.70	11.29	8.50	12.44	9.77	0.385	0.006	0.155	0.938
Neu, $\times 10^3$ cells/mm^3	3.31	1.95	4.40	2.59	5.11	3.10	0.243	0.003	0.192	0.411
Lymp, $\times 10^3$ cells/mm^3	6.69	5.44	6.37	5.63	6.65	5.96	0.226	0.126	0.614	0.370
NLR	0.54	0.36	0.69	0.46	0.77	0.51	0.036	0.009	0.686	0.605

[1] RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; WBC, white blood cell count; Neu, neutrophil; Lymp, lymphocyte; NLR, the ratio of neutrophil to lymphocyte.

Table 3.5 Effect of postbiotic derived from heat killed *Limosilactobacillus ingluviei* C37 supplementation on serum cortisol and plasma antioxidant capacity in calves during 1-89 days of age.

	Time							P value		
	Day 76		Day 83		Day 89					
Item[1]	CON	TRT	CON	TRT	CON	TRT	SEM	Group	Time	GxT
Cortisol, ng/mL	6.39	5.10	8.97	5.72	12.27	8.42	0.599	0.011	0.023	0.042
TAOC, U/L	4.36	4.54	3.89	4.09	3.57	3.93	0.132	0.338	0.307	0.825
SOD, U/L	17.12	17.31	22.17	24.11	19.23	20.87	0.728	0.220	0.021	0.439
GPX, U/L	33.38	34.37	48.67	54.02	30.74	40.04	2.169	0.085	0.002	0.030
CAT, U/L	26.56	34.81	39.74	45.89	21.71	28.03	1.884	0.002	0.001	0.658
MDA, nmol/mL	2.70	2.56	3.10	2.87	3.85	3.25	0.100	0.010	0.008	0.009
DPPHsc, μmol VC/mL	21.15	22.43	25.28	27.06	16.27	18.89	0.893	0.060	<0.001	0.483

[1] TAOC, total antioxidant capacity; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; DPPHsc, 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity.

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3.6 Discussion

3.6.1 Effect of postbiotic LIC37 supplementation affects growth performance in calves

Feed intake, ADG, and feed efficiency are key indicators for evaluating animal production performance. However, factors such as pathogenic and nutritional stressors directly influence feed intake, and growth performance in calves during the weaning period (Vi et al., 2004). Postbiotics have been shown to alleviate stress and improve growth performance (Kareem et al., 2016; Kim and Duarte, 2024). Thorsteinsson and Vestergaard (2020) found that *Saccharomyces cerevisiae* and a postbiotic from *Lactobacillus acidophilus* increased final body weight, ADG, and improved feed efficiency in veal calves.

In comparison, postbiotic LIC37 supplementation resulted in higher values of BW gain and TDMI especially after feeding starter (day 1-33), but the differences were not statistically significant. Interesting, different to stage of MR-only feeding, postbiotic LIC37 supplementation showed greater feed efficiency ($P < 0.05$) for the stage after feeding the starter. This may be related to the fact that the calves were exclusively fed easily digestible milk replacer at this stage. After the introduction of starter, the intake of solid feed promotes ruminal fermentation, thereby triggering the physical and metabolic development of the calf (Vi et al., 2004). Similarly, as reported by Izuddin et al. (2018), postbiotic can significantly improve feed efficiency by improving rumen development, optimizing gut microbiota, and enhancing feed digestion utilization. Additionally, Zhang et al. (2016) reported that *L. plantarum* and *B. subtilis* supplementation improved protein digestibility in Holstein calves, showing a significant improvement by week 8 compared to the control group, while no significant difference was observed at week 6. Regrettably, our present study has not included an analysis of apparent digestibility, we intend to conduct more comprehensive investigations and validation in subsequent research. On the other hand, as reported by Timmerman et al. (2005) the beneficial effect of probiotics might only manifest when an animal's health status was compromised. Therefore, we speculate that the difference may be related to the relief of stress caused by diet change and weaning by the addition of postbiotic. Notably, postbiotic improved feed efficiency, suggesting a potential economic benefit for the TRT group. This finding is consistent with previous research,

where improved feed efficiency was observed in lambs receiving postbiotic from *Lactobacillus plantarum* RG14 (Izuddin et al., 2019b). Similarly, Rius et al. (2022) reported that calves fed postbiotic from *Aspergillus oryzae* under heat stress exhibited a reduction in feed intake and a trend toward increased feed efficiency, although body weight gain remained unaffected.

3.6.2 Effect of postbiotic LIC37 supplementation on blood biochemical parameters in calves

Under stress conditions, proinflammatory factors such as IL-6, TNF-α, and IFN-γ typically increase (Zhang et al., 2018), stimulating B cell proliferation, which in turn elevates globulin levels (Vazquez et al., 2015). In addition, weaning stress increases intestinal permeability (Meale et al., 2017), leading to the entry of bacterial endotoxin (e.g. Lipopolysaccharide) into the blood proinflammatory cascade (Eckel and Ametaj, 2016), which increases the synthesis of acute phase proteins (APPs). In the present study, we observed elevated serum albumin and globulin levels in calves during weaning, which may be related to weaning stress (Hickey et al., 2003a). Kim et al. (2012) reported that feeding vitamin C reduced total protein and globulin in serum of calves during the summer growing period. Therefore, we speculate that the lower levels of total protein and globulin, along with the higher AGR observed in the TRT group, may indicate a reduction in the stress response due to postbiotic LIC37 supplementation during weaning. This might due to postbiotic LIC37 enhanced the intestinal barrier function (Zhang et al., 2022), and reduced the penetration of harmful microorganisms and toxins (Lázaro et al., 2024), thereby reducing the degree of immune activation of the body. These findings align with the previous study, Wang et al. (2022) found that feeding neonatal calves with low compound probiotics had decreased total protein and globulin levels.

Glucose serves as the main energy source for pre-weaning calves and is directly influenced by milk intake (Suarez-Mena et al., 2017). In the present study, postbiotic LIC37 supplementation did not affect blood glucose concentration, which aligns with the results of MR intake. After weaning, however, rumen development leads to a transition in the main energy source from glucose to VFA (Suarez-Mena et al., 2017), while liver metabolism transitions from glycolysis to gluconeogenesis (Donkin and Armentano, 1995). These changes collectively result in a decrease in blood glucose

concentration. Unexpectedly, we found weaning had no effect on serum glucose levels. This could be due to the specific weaning strategy employed, where sampling occurred the day after MR supply was reduced. BUN concentration is associated with decreased protein synthesis and catabolism (Wang et al., 2021), and is an indicator of amino acid utilization efficiency (Lv et al., 2018). Additionally, BUN is considered an indicator of rumen development in calves (Khan et al., 2007a). Elevated BUN levels reflect nitrogen inefficiency, resulting from an excessive supply of protein to the tissues (Melendez et al., 2003). Kazana et al. (2021) found that serum BUN levels in calves was significantly higher post-weaning compared to pre-weaning. Similarly, we observed weaning significantly increased serum BUN levels, which might be attributed to impair protein turnover (Deng et al., 2009). Notably, postbiotic LIC37 supplementation decreased serum BUN levels, indicating that postbiotic LIC37 improve nitrogen utilization in calves. Similar findings have been reported in previous studies. For example, Saleem et al. (2017) observed that weaning increases BUN concentration in lambs, while probiotic supplementation lowers BUN levels post-weaning. Likewise, Razavi et al. (2019) found that feeding dairy cattle with yeast cell wall reduced BUN concentration in serum.

Weaning is a stressful transition involving dietary changes from milk to solid feed, affecting lipid metabolism. Cholesterol synthesis in the liver is regulated by energy balance and metabolic stress (Brown et al., 2021), which may be altered during weaning. In the present study, we observed a reduction in cholesterol by the weaning period. This change may have resulted from a switch from high-fat milk substitutes to solid diets (Alhaidary et al., 2010). Additionally, stress may cause cortisol secretion (Hickey et al., 2003b), and inhibit liver cholesterol production (Fraser et al., 1999), thereby reducing blood cholesterol levels. On the other hand, microbial feed additives reduce lipid absorption in the intestines by deconjugation (Saleem et al., 2017). Consequently, feeding probiotics has the potential to lower serum cholesterol. For example, Naseem et al. (2024) reported that feeding growing lambs with probiotic reduced plasma cholesterol. Similarly, Saleem et al. (2017) demonstrated that probiotic not affect the level of blood glucose while decreasing serum cholesterol in post-weaning lambs. Since postbiotic is a preparation of inanimate microorganisms and/or their components (Salminen et al., 2021), we speculate that postbiotic and

probiotic have similar mechanisms in lowering blood cholesterol. Consistently, Humam et al. (2020) reported that feeding postbiotic from *Lactobacillus plantarum* reduced plasma cholesterol in broilers. Likewise, we observed a reduction in serum cholesterol levels with postbiotic LIC37 supplementation. Triglycerides are the primary form of lipid energy storage in animals (Allen, 1976). The synthesis and storage of serum triglycerides primarily occur in the liver and adipose tissue (Wang et al., 2011). A study by Khan et al. (2007b) reported that weaning led to a reduction in serum triglyceride levels. This finding is consistent with our study, in which we observed a decline in serum triglyceride concentrations both during and post-weaning. This reduction may be attributed to a decrease in fat absorption following the cessation of milk replacer feeding (Quigley et al., 1994). Interestingly, probiotic can regulate blood glucose and improve lipid metabolism by reducing triglyceride levels in metabolic disorders (Zavišić et al., 2022). Numerous studies have demonstrated it. For example, Dar et al. (2019) reported that feeding with *Lactobacillus acidophilus* reduced serum triglycerides levels in calves. Additionally, probiotic supplementation has resulted in similar reductions in serum triglycerides in dairy cows (Elbarbary et al., 2019), feedlot cattle (Mansilla et al., 2023), and ewes (Baiomy, 2010). Likewise, we observed a reduction in triglyceride by postbiotic LIC37 supplementation, indicating that postbiotic LIC37 may improve lipid metabolism in calves during the weaning period.

ALT and AST are commonly used as biomarkers of tissue damage. ALT is mainly synthesized in the liver, whereas AST is found in many tissues besides the liver (Rosenthal and Haight, 1990). When certain cell types are damaged, they leak enzymes into the blood (Magistrelli et al., 2013). Thus, increased ALT and AST levels often indicate tissue damage (Rosenthal and Haight, 1990). In the present study, we observed a significant increased ALT and AST levels during the weaning period ($P < 0.05$), which may indicate liver damage induced by weaning (Nagaraja et al., 2006). Interestingly, although postbiotic LIC37 supplementation did not affect ALT and AST levels, a significant interaction between postbiotic supplementation and weaning was observed for ALT levels ($P = 0.040$), suggesting that postbiotic LIC37 may influence ALT levels during a specific period.

3.6.3 Effect of postbiotic LIC37 supplementation on complete blood count in calves

HGB is the main component of RBC, directly participates in the transport of oxygen and carbon dioxide (Shah and Altindag, 2004). Weaning stress may activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased cortisol production (Masmeijer et al., 2021), which in turn may stimulate erythropoietin (EPO) to release additional HGB (Peschle et al., 1971), thereby accelerate oxygen transport. On the other hand, weaning stress can cause intestinal disorders like diarrhea, leading to dehydration (Probo and Veronesi, 2022), which often alters HCT, HGB, total protein, and electrolyte levels (Atata et al., 2019). Consistent with change in TP levels, weaning increased HGB ($P < 0.05$), and a numerical increase in HCT values was also observed ($P > 0.05$), suggesting that may mild dehydration in calves. However, no obvious diarrhea was observed during weaning. Unfortunately, analyses of EPO and electrolytes were not included in this study, and we intend to investigate and validate them more comprehensively in subsequent studies. Consistent with our results, Ayyat et al. (2023) reported that feeding *Lactobacillus plantarum* did not affect HGB levels, whereas the weaning period led to an increase in HGB levels.

Neutrophils are key effector cells of the innate immune system (Mayadas et al., 2014), serving as the first line of defense against pathogens (Paape et al., 2003). They respond to various signals by producing cytokines and inflammatory factors that regulate both inflammation and immune responses (Nauseef and Borregaard, 2014; Scapini and Cassatella, 2014). Weaning stress can activate the HPA axis in calves, leading to increased cortisol levels (Masmeijer et al., 2021), which subsequently trigger a systemic stress response and an increase in neutrophil level (Tang et al., 2022). In the present study, we observed reduction in neutrophil count and NLR, along with stable lymphocyte levels, suggests a potential attenuation of systemic inflammation and stress response in postbiotic-supplemented calves. This decline in neutrophil levels further echoes the previously decrease in globulin levels. This may be attributed to postbiotic LIC37 improved gut barrier function, and reduced microbial translocation, leading to decreased immune activation (Ji et al., 2023). These findings align with the previous study of Izuddin et al. (2019a), who reported that post-weaning lambs fed postbiotic from *L. plantarum* RG14 exhibited significantly lower WBC and neutrophil

levels, suggesting that postbiotics help modulate immune homeostasis by reducing unnecessary innate immune activation while maintaining adaptive immune functions.

3.6.4 Effect of postbiotic LIC37 supplementation on cortisol and antioxidant capacity in calves

Cortisol is a vital regulator of metabolism, influencing processes such as lipolysis, gluconeogenesis, and glycogenesis (Arfuso et al., 2023). Released in response to the activation of the hypothalamic-pituitary-adrenal (HPA) axis during stress, it helps the body adapt to challenges by modulating various physiological responses (Masmeijer et al., 2021). Expectedly, the weaning strategy in this experiment increased serum cortisol concentration, suggesting the calves were under stress. This finding is consistent with previous studies. For example, Agustinho et al. (2024) observed a significant changes in serum cortisol was observed in calves during the weaning period. Similarly results were found in dairy calves (Kim et al., 2011) and beef calves (O'Loughlin et al., 2014).

A stable levels of free radicals play a crucial role in intracellular signaling pathways and gene regulation by oxidizing specific transcription factors (Sena and Chandel, 2012). Normally, the endogenous antioxidant system usually activates enzymes to neutralize an excess of free radicals, thereby preserving redox balance (Ban et al., 2025). When this balance is disturbed, accumulated free radicals cause damage to cellular structure and function, leading to the peroxidation of unsaturated lipids and the formation of MDA (Akhalaya et al., 2006). Consistent with changes in cortisol, we found that MDA was elevated during weaning, a manifestation of lipid peroxidation. Similarly, Majlesi et al. (2021) demonstrated that MDA levels were significantly increased during calf weaning. On the other hand, we observed that the levels of certain antioxidant enzymes (e.g. SOD, GPX, and CAT) initially increased and then declined during the weaning period, which may reflect a compensatory response of the antioxidant system (Rizvi and Maurya, 2007). However, prolonged weaning stress may exacerbate oxidative stress, potentially leading to a decline in these antioxidant enzymes.

In the endogenous antioxidant system, SOD catalyzes the conversion of superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2), which is subsequently degraded into H_2O by CAT or GPX (Majlesi et al., 2021). In the present study, a

reduction in cortisol and MDA levels was observed in TRT group, suggesting that postbiotic plays an important role in alleviating calf stress and mitigating lipid peroxidation during weaning. Additionally, a significant increase in CAT levels, and along with a tendency for increased GPX level and DPPH scavenging capacity, was noted. This finding indicates that postbiotic LIC37 primarily enhance the endogenous antioxidant system while also facilitating the removal of oxidative damage products (e.g. MDA). Similar results have been reported in previous studies. For example, Izuddin et al. (2020) reported that postbiotic *Lactobacillus plantarum* RG14 increased GPX and reduced MDA levels in the serum of lambs. Likewise, a study on weaned piglets fed postbiotic from *Lactobacillus reuteri* observed elevated SOD levels and a tendency toward lower MDA level (Sun et al., 2025). Take together, these findings suggest that postbiotic LIC37 supplementation can enhance antioxidant defense mechanisms in calves, potentially alleviating oxidative stress and improving overall physiological resilience during this critical transition period.

3.7 Conclusions

In conclusion, the present study demonstrated that supplementation with postbiotic from heat killed *Limosilactobacillus ingluviei* C37 improved feed efficiency, suggesting a beneficial effect on nutrient utilization. Postbiotic LIC37 supplementation significantly reduced serum globulin and total protein levels, and increased the albumin-to-globulin ratio, indicating improved protein metabolism and possibly reduced systemic inflammation. Additionally, postbiotic supplementation significantly decreased cortisol and malondialdehyde (MDA) levels while enhancing catalase (CAT) activity. Furthermore, there was a tendency toward increased glutathione peroxidase (GPX) levels and free radical scavenging capacity (DPPH), indicating an improved antioxidant defense system. Immunologically, postbiotic LIC37 supplementation led to a significant reduction in white blood cell (WBC) count, neutrophils (Neu), and the neutrophil-to-lymphocyte ratio (NLR), further supporting its anti-inflammatory potential. Collectively, these findings suggest that postbiotic LIC37 supplementation supports metabolic stability, enhances antioxidant, and immune status during the critical weaning transition in dairy calves. Further investigations into long-term effects and dose optimization are warranted

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CHAPTER IV

EFFECT OF DIETARY POSTBIOTIC LIC37 ON LIVER TRANSCRIPTOMIC PROFILE OF CALVES

4.1 Abstract

This study aimed to investigate the effect of postbiotic from heat killed *Limosilactobacillus ingluviei* C37 (postbiotic LIC37) on liver transcriptional response of calves. Fourteen calves were assigned to 2 treatment groups: CON (n = 7) or TRT (n = 7, fed 108 CFU/d of inactivated Lactobacillus ingluviei CR37 strain). Calves received milk replacer (MR) at 1.75% of body weight (based on air-dry weight) and the amount was adjusted weekly according to body weight, while fresh and clean water was provided ad libitum. All calves had free access to starter feed starting on day 33. On day 82, the MR solution was reduced to 50% of the previous week's allocation, and calves were completely weaned by day 89. Liver samples were collected after slaughter on day 90. Transcriptome analysis identified 33 DEGs, including 16 upregulated DEGs such as Endothelial lipase (LIPG), Peroxisomal Acyl-CoA oxidase 1 (ACOX1), Solute carrier family 27 member 6 (SLC27A6), and 17 downregulated DEGs such as Family with sequence similarity 107 member A (FAM107A), 4-Hydroxy-2-oxoglutarate aldolase 1 (HOGA1), Farnesyl diphosphate synthase (FDPS). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified 11 significant pathways, including the PPAR signaling pathway and Pentose phosphate pathway. Taken together, postbiotic LIC37 supplementation demonstrated potential benefits in improving lipid metabolism and mitigating oxidative stress in weaning calves.

Keywords: Postbiotic, *Limosilactobacillus*; Calf weaning; Transcriptome; Liver; lipid metabolisms.

4.2 Introduction

Weaning is a critical and demanding transition for calves, frequently leading to physiological stress responses and disrupting energy homeostasis (Agustinho et al., 2024), which can compromise growth performance and overall health. As a key regulatory hub for overall energy metabolism, the liver plays a pivotal role in metabolic adaptation by modulating fatty acid uptake and release, de novo synthesis, and lipid utilization through β-oxidation, and oxidative stress responses (Badmus et al., 2022).

During weaning, nutrient intake of calves is insufficient, and ammonia absorbed by the rumen increases the burden on the liver through the liver urea cycle (Laarman et al., 2012; Batista et al., 2022). Additionally, excessive lipid accumulation in the liver exacerbates oxidative stress, enhances fatty acid oxidation, and triggers the release of proinflammatory cytokines, which collectively contribute to mitochondrial and hepatocyte damage, inflammation, and the activation of fibrotic pathways (Kutlu et al., 2018).

Dietary intervention optimizes energy balance by enhancing antioxidant defense and regulating lipid metabolism, offering a new approach to alleviate weaning stress in calves. Probiotics are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). In recent years, postbiotics, as an alternative to probiotics, have been extensively used to improve growth and lactation performance, as well as to enhance immunity and antioxidant defenses (Stefańska et al., 2022; Chae et al., 2024). For example, Ríus et al. (2022) reported postbiotic from *Aspergillus oryzae* improved energy-use efficiency in calves exposed to heat stress. Similarly, Dai et al. (2024) demonstrated that supplementing transition dairy cows with postbiotics from *Saccharomyces cerevisiae* could be beneficial for improving liver metabolism. Additionally, Izuddin et al. (2020) observed that the antioxidant enzymes concentration were increased by postbiotic *Lactobacillus plantarum* in post-weaning lambs. However, the molecular mechanisms through which postbiotics influence liver metabolism in weaned calves remain to be investigated. Recently, RNA-Seq technology efficiently identifies nearly all transcripts in tissue, thereby uncovering the molecular mechanisms underlying biological phenomena (Malone and Oliver, 2011).

4.3 Objective

This study aimed to investigate the effects of postbiotics from heat killed *Limosilactobacillus ingluviei* C37 on growth performance, antioxidant capacity, and liver transcriptomics in calves during the weaning period.

4.4 Materials and methods

4.4.1 Ethics statement

The experiments were carried out at the Suranaree University of Technology (SUT) farm according to the approved protocol by the Animal Care and Use Committee of SUT, Thailand (document no. SUT-IACUC-0020/2023).

4.4.2 Animals, treatment and sampling method

The *Limosilactobacillus ingluviei* C37 strain was obtained from the Laboratory of Monogastric Animal Nutrition and Feed Science at Suranaree University of Technology (SUT). The isolation and cultivation of *L. ingluviei* C37 were thoroughly detailed by Sirisopapong et al. (2023). The inactivated preparation of *L. ingluviei* C37 was achieved by heat-killing the cells at 80°C for 30 minutes, following the method described by Tsukagoshi et al. (2020).

Fourteen Holstein bull calves were obtained from a local dairy farm at the age of 5.71 ± 1.14 d. Each calf received 2 liters of colostrum within 3 hours of birth, followed by an additional 2 liters within the next 12 hours. All calves with serum total protein levels exceeding 5.6 g/dL at 24 hours of birth were selected, confirming the successful transfer of passive immunity (Hernandez et al., 2016). The calves were gradually transitioned from colostrum to bucket-fed milk replacer (MR) beginning at three days of age before being transferred to the SUT farm. Upon arrival, they received an immediate intramuscular injection of vitamin B12 (Catosal^{TM}, OLIC Co. Ltd, Ayutthaya, Thailand) at a 5% (mL/kg) dosage. All calves were blocked into 2 groups by body weight (37.34 ± 3.19 kg, and 28.83 ± 2.92 kg; mean ± SD), and randomly assigned to 2 treatments (7 per treatment). Namely, CON group (without postbiotic) and TRT group with 1 g/d of postbiotic LIC37 (10^8 CFU/g). The dosage was determined based on conversions from previous studies (Thorsteinsson et al., 2020; McNeil et al., 2024). Each calf was kept in an individually pen (2.2 m × 2.4 m) equipped with rubber mats

and wood pellets. The soiled wood pellets were removed daily, and fresh wood pellet was provided weekly.

Commercial MR was procured from Dairy-Rich Co. Ltd (Bangkok, Thailand), with its nutrient composition detailed in **Table 3.1** (Chapter III). The MR for calves was provided twice daily at 08:00 and 16:00 at a concentration of 15%, equivalent to 1.75% of BW (base on air-dry). The feeding amount was adjusted weekly based on BW. The postbiotic was mixed into the morning MR feeding, while fresh and clean water was available *ad libitum*. As the sole solid feed, the commercial starter (Charoen Pokphand Foods, Bangkok, Thailand) was provided *ad libitum* from day 33 of the experiment.

The 90-day feeding trial began on the day the calves arrived at SUT farm. The weaning process was completed within one week. Specifically, it started on day 82, with the MR solution reduced to 50% of the previous week's allocation. Calves were completely weaned by day 89. This feeding strategy was intended to induce weaning stress (Van Niekerk et al., 2021). On day 90, prior to the morning feeding, all calves were euthanized by captive bolt stunning and exsanguination. The liver tissue was taken from the caudate lobe into sterile RNase-free tubes, snap-frozen in liquid nitrogen, and then stored at -80°C for future RNA extraction.

4.4.3 RNA extraction and RNA-seq library construction

According to the manufacturer`s instructions, total RNA was obtained from liver tissue using TRIzol reagent (Molecular Research Center, Cincinnati, Ohio, USA). The quality and quantity of the extracted RNA were analyzed using spectrophotometry (NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA) and assessed by 1% agarose (w/v) gel electrophoresis, with 0.5 × TAE buffer and an applied electric current of 100 V for 20 minutes. RNA integrity was further evaluated using capillary electrophoresis on the QIAxcel Connect (Qiagen) to determine the RNA integrity number (RIN). RNA samples with a RIN > 8 were selected for the construction of RNA libraries.

RNA reverse transcription, library preparation, and RNA sequencing (RNA-seq) were carried out at BGI Co., Ltd. (Shenzhen, China). Briefly, total RNA was enriched for mRNA by poly(A) selection using oligo(dT) magnetic beads, followed by reverse transcription and cDNA synthesis. The resulting double-stranded cDNA underwent end repair, 5'-phosphorylation, and 3'-adenylation to prepare for adapter ligation. The adapter-ligated products were then PCR amplified, denatured, and circularized using bridging

primers to create single-stranded circular DNA libraries. Sequencing was performed on the DNBSEQ platform with PE500 (BGI Co., Ltd., Shenzhen, China). The raw sequencing data were processed using SOAPnuke (v1.5.6, RRID:SCR_015025) to eliminate low-quality and adapter-contaminated reads. The filtering criteria were as follows: 1) removal of reads containing adapter sequences, 2) exclusion of reads with more than 5% unknown bases (N), and 3) discarding low-quality reads, defined as those in which more than 20% of the bases had a quality score below 15.

4.4.4 Transcriptome sequencing, data analysis, and functional enrichment analyses

The high-quality reads were retained as clean data and subsequently analyzed using the online multi-omics data mining platform (biosys.bgi.com). In brief, The sequencing reads were mapped to the *Bos taurus* reference genome (GeneBank Assembly ID: GCA_002263795.2) using HISAT2 (version 2.2.1) with default settings (Kim et al., 2015). Cleaned data were then aligned to the reference transcriptome using Bowtie (version 2.3.4.3) (Langmead and Salzberg, 2012). Gene expression levels were quantified with RSEM (version 1.3.1) (Li and Dewey, 2011). Differential gene expression analysis between the two groups was performed using DESeq2 (version 1.4.5) (Love et al., 2014). The transcripts were filtered for sufficient normalized read depth, requiring transcripts per million greater than 0 (TPM > 0) in at least 5 samples per group.

Transcripts with a fold-change (FC) of ≥ 1 and an adjusted P value < 0.05 were considered differentially expressed genes (DEGs). Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses for DEGs were performed using the Phyper function in R software. GO terms and KEGG pathways with a P value < 0.05 were performed regarded as significantly enriched. All sequencing data have been deposited in the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) database under the accession number GSE293736.

4.4.5 Quantitative polymerase chain reaction to validate DEGs

To confirm the reliability and precision of gene expression data obtained from RNA-Seq, quantitative PCR (qPCR) was conducted on the same RNA samples. The RNA was reverse transcribed into cDNA using SweScript All-in-One RT SuperMix (G3337, Servicebio Technology Co., Ltd., Wuhan, China) according to the manufacturer's instructions. Using Primer3 software (https://primer3.ut.ee/) designed primers (**Table 4.1**), and subsequently

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synthesized by Servicebio Technology Co., Ltd. (Wuhan, China). Five target genes were Endothelial lipase (LIPG), Acyl-CoA oxidase 1 (ACOX1), Family with sequence similarity 107 member A (FAM107A), 4-Hydroxy-2-Oxoglutarate aldolase 1 (HOGA1), and Farnesyl diphosphate synthase (FDPS). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was housekeeping gene in this study. The qPCR was performed on CFX Connect™ Real-Time PCR System (Bio-Rad, California, USA) in a reaction solution (15 µL) contained 2 µL of cDNA template, 1.5 µL of each primer (10 µM), 7.5 µL of 2×Universal Blue SYBR Green qPCR Master Mix (G3326, Wuhan Servicebio Technology Co., Ltd), and 4 µL of nuclease-free water. The qPCR conditions were as follows: predegeneration at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 60°C for 30 seconds. Relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Love et al., 2014), and the resulting values were transformed into fold change (FC) to enable comparison with RNA-Seq data.

Table 4.1 Primer sequences used in quantitative PCR.

Gene	Primer sequences (5' to 3')	Accession
LIPG	(F): TCA AGC CCC TTC ACA TTC CC	XM_002697766
	(R): CTC TCG AAG TTT CCA GCG GT	
ACOX1	(F): GCG TTA CGA GGT GGC TGT TA	NM_001035289
	(R): GGC CCA CAG GTT CCA CAA AA	
FAM107A	(F): CTG AGA ACG CAG GAC CCG	XM_024982918
	(R): AGC AGC TTC TTG GGC TTG AT	
HOGA1	(F): GAG GTG GAC TAT GGG AAA CTG G	XM_024985530
	(R): CCT CTG AAC TTC TCT TGC CTG T	
FDPS	(F): GAG GCA GGG GCT AGA AAC TC	XM_024984593
	(R): ATT CCC AAA ACG GGG GAA CA	

LIPG, Endothelial lipase; ACOX1, Peroxisomal Acyl-CoA oxidase 1; FAM107A, Family with sequence similarity 107 member A; HOGA1, 4-hydroxy-2-oxoglutarate aldolase 1; FDPS, Farnesyl diphosphate synthase.

4.5 Results

4.5.1 RNA-Seq data processing and quality control

According to the results presented in **Table S4.1**. RNA sequencing generated an average of 46.82 million raw reads from 14 libraries, with minimum of 45.44 million to a maximum of 47.19 million. After quality control, an average of 45.03 million clean reads were obtained, with the number of clean reads ranging from a minimum of 43.84 million to a maximum of 45.51 million. A minimum of 95.74% of the reads demonstrated a sequence quality score above Q30. The total Mapping rates ranged from 98.29% to 98.66%, and the average unique mapping rate was 95.01%.

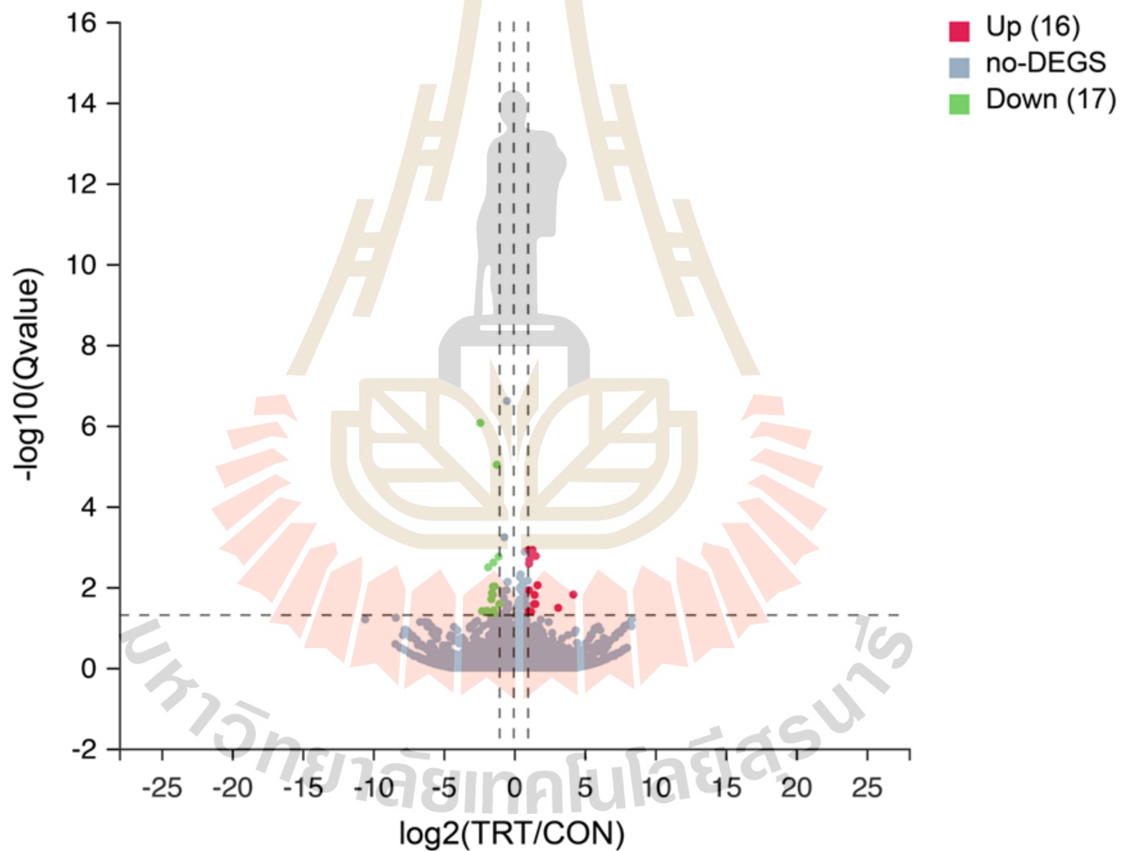

Figure 4.1 Volcano plot of differentially expressed genes in the liver tissue of calves. The red and green dots representing upregulated and downregulated transcripts, respectively. Gray dots represent insignificant DEGs. The x and y axes of the volcano plots show the log2 fold changes and –log10 q value, respectively.

4.5.2 Detection of DEGs

As presented in **Figure 4.1**. A total of 33 DEGs were identified in liver tissue, with 16 upregulated and 17 downregulated transcripts. Detailed information about the identified DEGs is provided in **Table S 4.2**.

Among these, Endothelial Lipase (LIPG), Peroxisomal Acyl-CoA oxidase 1 (ACOX1), Solute carrier family 27 member 6 (SLC27A6), Elongation of very long chain fatty acids protein 6 (ELOVL6), Glycerate kinase (GLYCTK), 4-Hydroxy-2-oxoglutarate aldolase 1 (HOGA1), Exostosin like glycosyltransferase 1 (EXTL1), and Farnesyl diphosphate synthase (FDPS) were indentified. The top 10 upregulated and down regulated DEGs shown in **Table 4.2**.

Table 4.2 Top 10 upregulated and downregulated differentially expressed genes in the liver tissue of calves.

Gene ID	Gene Symbol	log2 fold change	Qvalue[1]	Regulated[2]
509808	LIPG	4.22	0.0155	Up
781161	FREM3	3.14	0.0330	Up
513996	ACOX1	1.69	0.0092	Up
537062	SLC27A6	1.55	0.0017	Up
613923	DYM	1.53	0.0266	Up
533333	ELOVL6	1.47	0.0158	Up
534842	ROBO2	1.45	0.0266	Up
524334	L3MBTL3	1.34	0.0016	Up
507949	GLYCTK	1.32	0.0012	Up
525346	NCOA1	1.22	0.0414	Up
538515	FAM107A	-2.38	0.0000	Down
506001	HOGA1	-2.25	0.0397	Down
782061	LOC782061	-1.92	0.0387	Down
281156	FDPS	-1.83	0.0033	Down
505584	FXYD5	-1.68	0.0444	Down
785762	DDH3	-1.60	0.0448	Down

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Table 4.2 Continue.

Gene ID	Gene Symbol	log2 fold change	Qvalue[1]	Regulated[2]
112442367	-	-1.59	0.0202	Down
786492	LGALS3	-1.55	0.0143	Down
507550	-	-1.47	0.0155	Down
281240	IGF2	-1.47	0.0099	Down

[1] LIPG, Endothelial lipase; FREM3, FRAS1 related extracellular matrix protein 3; ACOX1, Peroxisomal Acyl-CoA oxidase 1; SLC27A6, Solute Carrier Family 27 Member 6; DYM, Dymeclin; ELOVL6, Elongation of very long chain fatty acids protein 6; ROBO2, Roundabout guidance receptor 2; L3MBTL3, L3MBTL transcriptional repressor 3; GLYCTK, Glycerate kinase; NCOA1, Nuclear receptor coactivator 1; FAM107A, Family with sequence similarity 107 member A; HOGA1, 4-hydroxy-2-oxoglutarate aldolase 1; LOC782061, Aldo-keto reductase family 1, member C1-like; FDPS, Farnesyl diphosphate synthase; FXYD5, FXYD domain containing ion transport regulator 5; DDH3, Dihydrodiol dehydrogenase 3; LGALS3, Galectin 3; IGF2, Insulin-like growth factor 2.

[2] Q value is adjusted of P value.

4.5.3 GO and KEGG pathway enrichment of DEGs

Gene Ontology (GO) analysis categorized these DEGs into three functional groups: biological processes (BP), molecular functions (MF), and cellular components (CC). Specifically, the significant DEGs were annotated into 50 CC terms, among which 12 were significantly enriched (**Figure 4.2**). Additionally, they were mapped to 193 BP terms, with 116 showing significant enrichment (**Figure 4.2**), and 97 MF terms, of which 50 were significantly enriched (**Figure 4.3**).

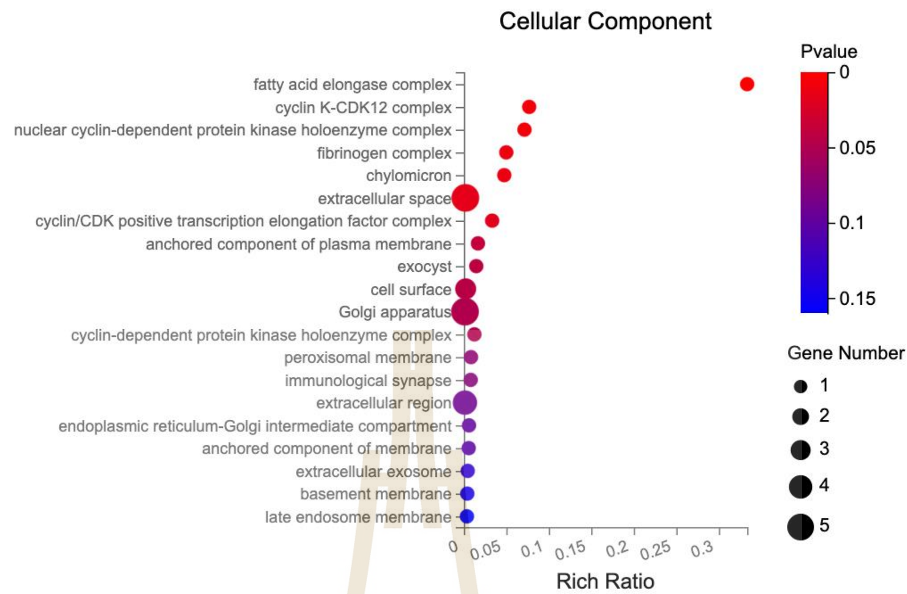

Figure 4.2 Top 20 GO cellular component terms enriched in differentially expressed genes in liver tissue in calves. The circle size in each term corresponds to the number of genes. The circle's color goes from blue to red, indicating a lower P value.

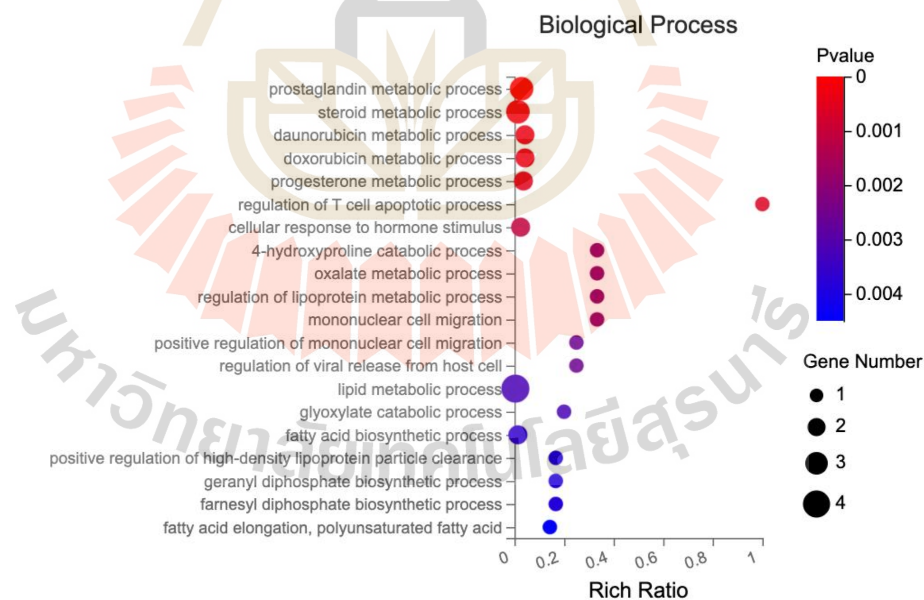

Figure 4.3 Top 20 GO biological process terms enriched in differentially expressed genes in liver tissue in calves.

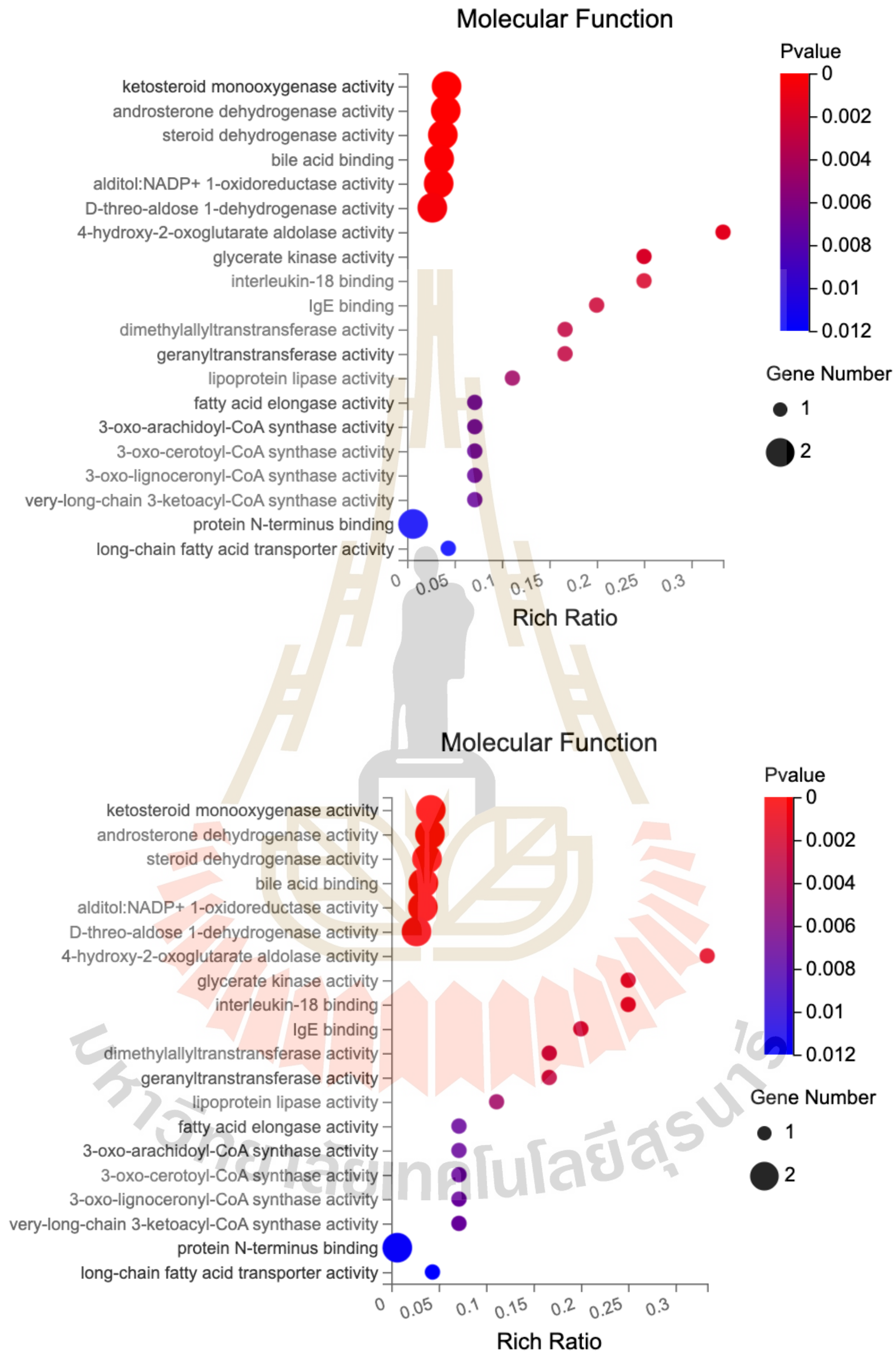

Figure 4.4 Top 20 GO Molecular function terms enriched in differentially expressed genes in liver tissue in calves.

A total of 47 KEGG pathways were identified from 33 DEGs, among which 11 pathways were significant enriched (**Table 4.3**). These include the PPAR signaling pathway (bta03320, P = 0.0109), Fatty acid metabolism (bta01212, P = 0.0037), Carbon metabolism (bta01200, P = 0.0105), Biosynthesis of unsaturated fatty acids (bta01040, P = 0.0005), Terpenoid backbone biosynthesis (bta00900, P = 0.0294), Glyoxylate and dicarboxylate metabolism (bta00630, P = 0.0007), alpha-Linolenic acid metabolism (bta00592, P = 0.0357), Glycerolipid metabolism (bta00561, P = 0.0058), Glycosaminoglycan biosynthesis - heparan sulfate / heparin (bta00534, P = 0.0315), Fatty acid elongation (bta00062, P = 0.0310), and the Pentose phosphate pathway (bta00030, P = 0.0456).

Table 4.3 Kyoto encyclopedia of genes and genomes pathways possibly affected by postbiotic in liver tissue of calves.

Pathway Term	Count	P value	Gene Symbols[1]
bta03320: PPAR signaling pathway	2	0.0109	ACOX1↑, SLC27A6↑
bta01212: Fatty acid metabolism	2	0.0037	ACOX1↑, ELOVL6↑
bta01200: Carbon metabolism	2	0.0105	ACOX1↑, GLYCTK↑
bta01040: Biosynthesis of unsaturated	2	0.0005	ACOX1↑, ELOVL6↑
bta00900: Terpenoid backbone	1	0.0294	FDPS↓
bta00630: Glyoxylate and	2	0.0007	GLYCTK↑, HOGA1↓
bta00592: alpha-Linolenic acid	1	0.0357	ACOX1↑
bta00561: Glycerolipid metabolism	2	0.0058	LIPG↑, GLYCTK↑
bta00534: Glycosaminoglycan	1	0.0315	EXTL1↓
bta00062: Fatty acid elongation	1	0.0310	ELOVL6↑
bta00030: Pentose phosphate	1	0.0456	GLYCTK↑

Up and down arrows indicate the upregulated and downregulated genes, respectively, in liver tissue of calves fed postbiotic LIC37.

4.5.4 qPCR validated RNA-seq results

We validated the expression levels of five transcripts, including two upregulated transcripts (e.g. ACOX1 and LIPG) and three downregulated transcripts (e.g. FAM107A, HOGA1, and FDPS), were quantified in liver tissue. All transcripts showed similar

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expression trends in both qPCR and RNA-seq (**Figure 4.3**). These results validate the accuracy of the identified transcripts and support the reliability of the RNA-seq results.

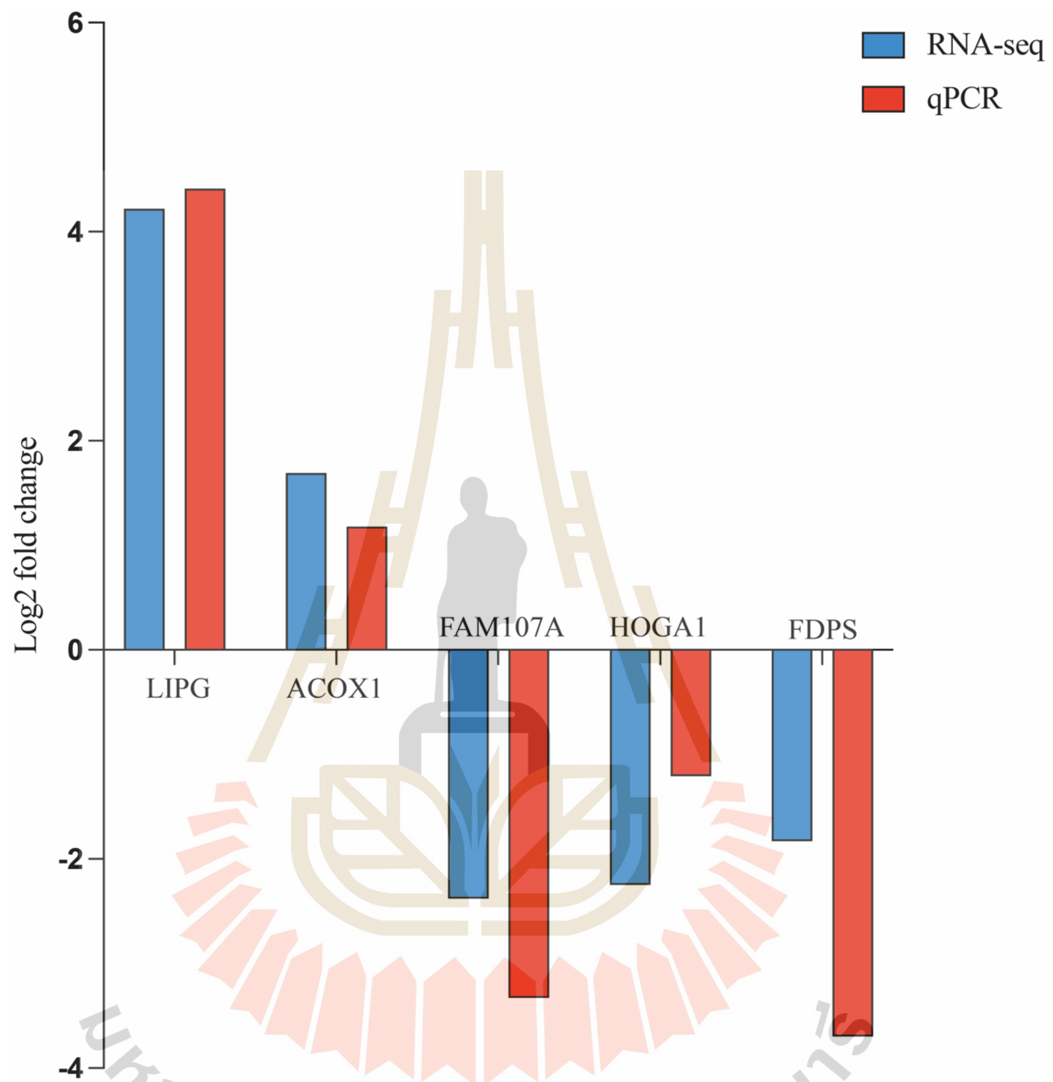

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Figure 4.5 Quantitative PCR (qPCR) was employed to validate five DEGs in RNA-seq analysis. The x-axis denotes the genes, while the y-axis shows their mRNA expression levels as fold-change (FC) values. Expression levels obtained from RNA-seq and qPCR are illustrated by blue and red bars, respectively. LIPG, Endothelial lipase; ACOX1, Peroxisomal Acyl-CoA oxidase 1; FAM107A, Family with sequence similarity 107 member A; HOGA1, 4-hydroxy-2-oxoglutarate aldolase 1; FDPS, Farnesyl diphosphate synthase.

4.6 Discussion

LIPG, ACOX1, and SLC27A6 are key regulators of lipid metabolism, contributing to fatty acid hydrolysis, oxidation, and transport. These genes contribute collectively to lipid homeostasis and energy metabolism. LIPG, a member of the triglyceride lipase family, is one of the key regulators of glyceride hydrolysis while directly facilitating the release of free fatty acids from phospholipids (Zhou et al., 2021) and high density lipoproteins (Strauss et al., 2003), playing a crucial role in maintaining cell structure, regulating cytokine expression, and providing energy (Hong et al., 2021). In the present study, LIPG expression in the TRT group had a FC of 4.22 greater than the CON group, suggesting that postbiotic LIC37 potentially enhanced glyceride hydrolysis and increased the release of free fatty acids. This may imply that postbiotic potentially enhances lipid mobilization and contributes to energy homeostasis in weaned calves.

ACOX1 is an essential enzyme in the acyl-CoA oxidase family and a key rate-limiting enzyme in peroxisomal β-oxidation (Shi et al., 2025). It facilitates the degradation of long-chain, branched-chain, and medium-chain fatty acids by promoting peroxisomal β-oxidation (He et al., 2020). The upregulation of ACOX1 in the TRT group observed in the present study suggests that postbiotic LIC37 may activate peroxisomal β-oxidation, which in turn promotes fatty acid degradation and enhances liver energy production in weaned calves. This result is consistent with previous finding by Wang et al. (2019), who demonstrated that *L. frumenti* administration upregulated ACOX1 protein, highlighting its role in activating fatty acid β-oxidation in the liver of early-weaned piglets. Thus, the upregulation of ACOX1 suggests a beneficial effect of postbiotic LIC37 on fatty acid metabolism, which may support the energy demands during the weaning process.

SLC27A6, is a member of the fatty acid transport family, and plays specific roles in fatty acid homeostasis (Bonen et al., 2007). As a membrane-associated fatty acid-binding protein, it regulates fatty acid transport, thereby influencing its utilization for triglyceride synthesis within the cell (Zhang et al., 2021). Additionally, SLC27A6 impacts lipid accumulation in tissues and cells by modulating fatty acid lipidation and oxidation (Huang et al., 2021). This finding is in line with previous reports by Nafikov et al. (2013), who reported that increased SLC27A6 expression promoted the uptake of fatty acids from plasma and the intracellular transport of fatty acids by mammary epithelial cells.

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Collectively, these findings indicate postbiotic LIC37 may promote fatty acid absorption and intracellular transport, potentially influencing lipid metabolism and energy distribution.

FDPS and HOGA1 are involved in lipid storage and adipogenesis. FDPS is a key enzyme involved in cholesterol and sterol biosynthesis (Claire D'Andre et al., 2013), playing a critical role in cholesterol and steroid metabolism by producing farnesyl diphosphate (Szkopińska and Płochocka, 2005). Downregulation of FDPS has been shown to suppress adipocyte differentiation in chickens (Zhu et al., 2023). The downregulation of FDPS inhibits intramuscular adipose differentiation and reduces energy storage, suggesting that postbiotic LIC37 may regulates energy redistribution in calves during weaning period. Similarly, HOGA1 is involved in glyoxylate and dicarboxylic acid metabolism, arginine and proline metabolism and other pathways to promote adipogenesis (Ye et al., 2024). Inhibition of HOGA1 has been shown to downregulate the expression of PPARγ, C/EBPα, AP2, CD36, and adiponectin during mouse adipocyte differentiation, thereby suppressing intracellular fat deposition (Kim et al., 2022). The downregulation of HOGA1 in the TRT group suggests that postbiotic LIC37 may inhibit adipogenesis and reduce liver fat accumulation, potentially shifting energy utilization away from lipid storage toward other metabolic processes.

FREM3 and FAM107A contribute to cell integrity and metabolic adaptation. FREM3 is a basement membrane protein of the Fras1-related extracellular matrix family, playing a crucial role in maintaining embryonic epithelial–mesenchymal integrity (Pavlakis et al., 2011). The upregulation of FREM3 by postbiotic LIC37 suggests that postbiotic LIC37 contributes to the maintenance of cell integrity. FAM107A has been shown to regulate the expression of CRYAB (Manigandan et al., 2021), which in turn modulates the CRYAB/PI3K/AKT signaling axis, a critical pathway involved in cell survival and metabolism (Ke et al., 2022). Ming and Zhang (2025) demonstrated that FAM107A depletion reduces CRYAB expression and increases PI3K and AKT phosphorylation, while FAM107A overexpression disrupts metabolic processes, decreasing glucose uptake, lactate production, and ATP levels. The downregulation of FAM107A in the TRT group observed in this study may suggest that postbiotic LIC37 supplementation enhances cellular metabolism, improving glucose uptake and ATP levels, thereby alleviating the insufficient energy supply in calves during weaning.

FXYD5 is implicated in inflammation and extracellular matrix regulation. As a type I plasma membrane protein, can promotes inflammation through TNFα signaling in normal cells (Lubarski-Gotliv et al., 2016). Its expression is associated with cytoskeletal reorganization (Schüler et al., 2012), altered cell shape (Shimamura et al., 2004), and the disruption of tight and adherence junctions (Miller and Davis, 2008; Lee et al., 2012). Furthermore, increased FXYD5 expression may exacerbate inflammation, oxidative stress, and extracellular matrix degradation by activating the NF-**K**B signaling pathway (Song et al., 2022). In the present study, a lower expression of FXYD5 in the TRT group compared with CON group, suggests a potential associated with a reduction in inflammation, oxidative stress, and extracellular matrix degradation during the weaning process in calves. This reduction in FXYD5 expression may indicate a less severe inflammatory response, lower oxidative stress levels, and improved tissue integrity in the TRT group. This finding is further supported by the observed increase in plasma antioxidant enzyme activity.

At the transcriptomic level, activation of the PPAR signaling pathway plays a crucial role in fatty acid biosynthesis and metabolism (Zhou et al., 2016), the maintenance of energy balance (Dupont et al., 2012), and the regulation of fatty acid oxidation (Nakagawa et al., 2016). Xu et al. (2022) found that glycyrrhizic acid and compound probiotics improved intestinal fat digestion and absorption by regulating PPAR signaling pathway in weaned piglets. Similarly, Cao et al. (2019) demonstrated that *L. plantarum* WW fermented soybean extract could improve fatty liver in rats *via* PPAR signaling pathway. Additionally, Zang et al. (2024) suggested that PPAR may be a potential target of *Lactobacillus plantarum*, which exerts anti-inflammatory effects by binding to p65/p50 to inhibit NF-**K**B activity. Similarly, the present study indicates that the PPAR/ACOX1/SLC27A6 may serve as a key regulatory target of a postbiotic LIC37 in liver lipid metabolism. Specifically, the postbiotic upregulates the transcription of ACOX1 and SLC27A6 *via* activating PPAR signaling pathway. ACOX1 enhances β-oxidation, promoting lipid degradation to meet the metabolic energy demands of weaned calves, while SLC27A6, a fatty acid transport protein, facilitates the uptake and utilization of long-chain fatty acids, further optimizing energy supply. Besides, lipid metabolism-related pathways, including fatty acid metabolism, biosynthesis of unsaturated fatty acids, fatty acid elongation, which function in fatty acid modification,

elongation, and energy production. Furthermore, we found that the postbiotic LIC37 may regulate fatty acid production and energy supply by activating fatty acid metabolism, biosynthesis of unsaturated fatty acids, and fatty acid elongation pathways. Additionally, we found that DEGs were enriched in oxidative stress-related pathways such as alpha-linoleic acid metabolism, glycerolipid metabolism, glyoxylate and dicarboxylate metabolism, and pentose phosphate pathway. Among these, pentose phosphate pathway is the central glucose catabolic pathways that link glucose metabolism to ribose synthesis and NADPH production (Huang et al., 2019), providing reducing power to the glutathione antioxidant system to scavenge reactive oxygen species (ROS) and protect cells from oxidative damage (Winkler et al., 1986).

Overall, we speculated that postbiotic LIC37 may improve energy supply by regulating lipid metabolism through PPAR signaling pathway, fatty acid metabolism, biosynthesis of unsaturated fatty acids, and the fatty acid elongation. Additionally, the postbiotic may alleviate oxidative stress damage through activation of alpha-linoleic acid metabolism, glycerolipid metabolism, glyoxylate and dicarboxylate metabolism, and pentose phosphate pathway, thereby mitigating the negative effects of weaning stress on calves.

4.7 Conclusion

In conclusion, RNA-seq analysis showed that 33 DEGs with 16 upregulation and 17 downregulation. KEGG pathway analysis revealed 11 significantly enriched pathways, among which heat killed *Limosilactobacillus ingluviei* C37 may regulate lipid metabolism *via* PPAR signaling pathway, and mitigate oxidative stress through pentose phosphate pathway. These findings highlight the potential of heat killed *Limosilactobacillus ingluviei* C37 to improve lipid metabolism and alleviate oxidative stress in calves during weaning.

4.8 References

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CHAPTER V

TRANSCRIPTOME ANALYSIS REVEALS JEJUNAL MECHANISMS UNDERLYING POSTBIOTIC LIC37 MEDIATED IMMUNE SYSTEM IN CALVES

5.1 Abstract

Weaning is a challenging for dairy calves, frequently resulting in digestive issues. This highlights the importance of implementing appropriate nutritional strategies to enhance gut health and support optimal growth. Postbiotics is a promising alternative to traditional probiotics by conferring health benefits without the risks associated with live bacteria. This study aimed to investigate the effect of dietary supplementation with postbiotic from heat killed *Limosilactobacillus ingluviei* C37 (postbiotic LIC37) on blood biochemical parameters, and jejunal epithelium transcriptomic profiles in calves. Results indicated that heat killed *Limosilactobacillus ingluviei* C37 supplementation reduced globulin, total protein, Neutrophil (Neu) and NLR levels in the TRT group ($P < 0.05$). Transcriptomic analysis identified 76 DEGs, with significant upregulation of genes involved in fatty acid metabolism (FABP1), intestinal barrier function (B4GALNT2), and detoxification (GSTA1), alongside downregulation of immune response regulation (FCRLA, FCRL4). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses highlighted enrichment in pathways related to Glutathione metabolism, Drug metabolism, and Vitamin digestion, indicating postbiotic LIC37 supplementation improved detoxification, oxidative stress defense, and nutrient absorption in calves. This study provides novel insights into the molecular mechanisms underlying the benefits of postbiotic LIC37 and supports their potential as a sustainable alternative to probiotics in calf nutrition.

Keywords: Calf weaning; Differentially expressed gene; Jejunal epithelium; *Limosilactobacillus*; Postbiotic; Transcriptome.

5.2 Introduction

Weaning is a particularly stressful and difficult event for dairy calves, which can easily cause intestinal disorders, with a high prevalence primarily attributed to developmental factors during the early postnatal stages. Specifically, gut immaturity, immune underdevelopment, and pathogen exposure primarily increase early life intestinal disease risk (Lopez and Heinrichs, 2022). Moreover, intestinal disorders not only compromise calf health; it also adversely affect weaning weights and subsequent milk yields (Aghakeshmiri et al., 2017). Hence, ensuring intestinal health is key to maximizing both growth potential and production performance.

Dietary intervention could offer novel approaches to ameliorate inflammatory disorders by modulating the immune response through metabolic rewiring. For decades, probiotics have been a staple in animal feed as additives to improve intestinal health (Mahesh et al., 2021). Yet, the widespread presence of antibiotic resistance genes in these strains, and the demonstrated ability for these genes to transfer between organisms (Marteau and Shanahan, 2003), casts a long shadow over their suitability for continued use as live bacteria in future nutritional strategies. Offering a compelling alternative to traditional probiotics, postbiotics are defined as a "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021), which could enhance host antioxidant capacity and immunity, regulate gut microbiota, and thereby support intestinal health. For example, Izuddin et al. (2019) found that feeding lambs with postbiotic from metabolites of *Lactobacillus plantarum* RG14 could improve growth performance, and nutrient intake; Feng et al. (2022) demonstrated that the profound effects of postbiotic from *Bifidobacterium bifidum* B1628 on alleviating inflammation and intestinal damage in murine models.

RNA sequencing (RNA-Seq) is widely utilized to examine transcriptomic changes in key tissues in response to various intrinsic and extrinsic factors, offering insights into gene regulation and physiological adaptations (Kim et al., 2022). Currently, RNA-Seq studies in ruminant nutrition mainly focus on the rumen (Zhao et al., 2017), colon (Jin et al., 2024), and liver (Fernandes et al., 2024), while studies investigating the jejunal epithelium in calves are relatively scarce.

5.3 Objective

This study aimed to conducted RNA-seq on the jejunal epithelium of calves to identify differential gene expression (DEGs) and uncover the underlying molecular mechanisms associated with a postbiotic from heat killed *Limosilactobacillus ingluviei* C37 supplemented diet.

5.4 Materials and methods

5.4.1 Ethics statement

The experiments were carried out at the Suranaree University of Technology (SUT) farm according to the approved protocol by the Animal Care and Use Committee of SUT, Thailand (document no. (SUT-IACUC-0020/2023).

5.4.2 Treatments, and management

The *Limosilactobacillus ingluviei* C37 strain (*L. ingluviei* C37) was sourced by the Laboratory of Monogastric Animal Nutrition and Feed Science at Suranaree University of Technology (SUT). The isolation and culture of *L. ingluviei* C37 was described in detail by report of Sirisopapong et al. (2023). The inactivated *L. ingluviei* C37 was prepared by heat-killed at 80°C for 30 min according to the method of Tsukagoshi et al. (2020).

Fourteen Holstein bull calves were collected from a single farm within one week of birth. The calf received 2 liters of colostrum within 3 hours of birth, and an additional 2 liters within the subsequent 12 hours. At 24 hours after birth, the serum total protein was using a Brix refractometer (Lohand Bio Co. Ltd. Hangzhou, China) to ensure an effective transfer of passive immunity (Hernandez et al., 2016), the calf with serum total protein greater than 5.6 g/dL was selected. Subsequently, the transition from colostrum feeding to bucket-fed milk replacer (MR) begins at 3 days of age, then transferred to the SUT farm. All calves received an intramuscular injection of 5% (v/w, mL/kg) vitamin B_{12} (Catosal^{TM}, OLIC Co. Ltd, Ayutthaya, Thailand) upon their arrived. Commercial MR was procured from Dairy-Rich Co. Ltd (Bangkok, Thailand). One kilogram of MR contained 974 g dry matter, 88.7 g ash, 226.7 g crude protein, and 177.4 g fat.A total of fourteen calves (33.69 ± 5.28 kg; mean ± SD) with similar age (5.71 ± 1.14 d; mean ± SD) were selected and randomly assigned to two groups (7 per group),

including 1 control group (without postbiotic LIC37), and 1 treatment group with 1g postbiotic LIC37 (10^8 CFU/g). The dose was converted based on previous studies (Thorsteinsson et al., 2020; McNeil et al., 2024). Each calf was housed in an individual pen (2.2 m × 2.4 m) with rubber mats and wood pellets on the floor. The contaminated wood pellets were cleaned daily, and the wood pellets were replaced weekly.

MR was fed daily at a concentration of 15% at 1.75% body weight (BW) (air-dry basis) at 08:00 and 16:00. MR was adjusted weekly for BW. The postbiotic LIC37 was mixed with MR during the morning feeding, and cleanly water was available *ad libitum*. As the sole solid feed, the commercial starter (Charoen Pokphand Foods, Bangkok, Thailand) was provided *ad libitum* from day 33 of the experiment. One kilogram of starter contained 909.4 g dry matter, 89.7 g ash, 237.9 g crude protein, 39.9 g fat, 520.9 g of neutral detergent fiber, and 122.4 g acid detergent fiber.

The experiment spanned 90 days, with the calves' arrival day considered as day 1. Daily recording of MR and starter intake, and measured BW weekly. The milk replacer was adjusted according to BW weekly. Calves began the weaning step-down at d 82, with the MR solution being restricted to 50% of the previous week's allocation. Calves were completely weaned by d 90. This feeding regimen was designed to elicit weaning stress (Van Niekerk et al., 2021).

5.4.3 Sampling method

On d 76 (pre-weaning), 83 (mid-weaning), and 90 (post-weaning), respectively. Prior to the morning feeding, blood samples were collected by jugular venipuncture using sterile tube without anticoagulation. Then, transferred to the SUT hospital detecting serum biochemical parameters, which encompassed total protein, globulin, albumin, and complete blood count.

On the last day of the experimental period (d 90), prior to morning feeding. Four calves were randomly selected from each group and euthanized using captive bolt stunning and exsanguinated. The abdominal cavity was quickly opened. The jejunum was defined as starting at 100 cm caudal to the pylorus. Jejunal samples were collected approximately 30 cm proximal to the collateral branch of the cranial mesenteric artery, rinsed three times with sterile phosphate-buffered saline (PBS, pH = 7.0), and immediately placed into sterile RNase-free tubes. The samples were then flash-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

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5.4.4 Library construction and data processing

Construction of the cDNA library and RNA-seq were performed by BGI Co. Ltd. (BGI, Shenzhen, China). Six libraries were tested on the DNBSEQ platform. Sequencing data were filtered using SOAPnuke Version v1.5.6 (Cock et al., 2010). Reads containing adapters, reads with unknown base N content greater than 5%, and low-quality reads (reads with a base quality value less than 15 accounting for more than 20% of the total base number of the reads) were removed to obtain clean reads. Subsequently, we used HISAT2 v2.1.0 (Kim et al., 2015) to align the clean reads to the chicken reference genome (GCF_000002315.6_GRCg6a) and then used RSEM Version v1.3.1 (Li and Dewey, 2011) to align the clean reads to the reference gene set.

5.4.5 RNA extraction and RNA-seq library construction

Total RNA was extracted from jejunal epithelium using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. The quality and quantity of the extracted RNA were analysis using spectrophotometry (NanoDrop 2000 spectrophotometer; Thermo Fisher Scientific, Waltham, MA) and 1% agarose (w/v) gel electrophoresis with 0.5 × TAE as a buffer and an electric current of 100 V for 20 min. The RNA integrity numbers (RIN) was determined by the capillary electrophoresis with a QIAxcel Connect, all the samples with RIN greater than 6.5 were used to construct the sequencing library.

RNA reverse transcription, library preparation, and RNA sequencing (RNA-seq) were conducted at BGI Co., Ltd. (Shenzhen, China). In brief, total RNA was subjected to mRNA with poly(A) enrichment using oligo(dT) magnetic beads for reverse transcription and cDNA synthesis. The double stranded cDNA underwent end repair, 5'-phosphorylation, and 3'-adenylation to facilitate adapter ligation. Adapter ligated products were PCR amplified, denatured, and circularized using bridging primers to construct single stranded circular DNA libraries. The sequencing was performed using the DNBSEQ platforms with PE500 (BGI Co. Ltd., Shenzhen, China).

The raw sequencing data were processed using SOAPnuke (v1.5.6, RRID:SCR_015025) to remove low-quality and adapter-contaminated reads. The reads were filtered based on the following criteria: 1) reads containing adapter sequences (adapter contamination), 2) reads with unknown bases (N) exceeding 5% of the total bases, and 3)

low-quality reads (defined as reads where more than 20% of the bases had a quality score below 15).

5.4.6 Transcriptome sequencing and data analysis

The resulting high-quality reads were retained as clean data and subsequently analyzed using the online multi-omics data mining system (biosys.bgi.com). In brief, the data were aligned to the reference *Bos taurus* genome (GeneBank Assembly ID: GCA_002263795.2) using HISAT2 version 2.2.1 (Kim et al., 2015) with default parameters. The clean data were aligned to the reference gene set using Bowtie version 2.3.4.3 (Langmead and Salzberg, 2012). The RSEM version 1.3.1 (Li and Dewey, 2011) was used to perform gene expression quantification. Differential expression analysis between the two groups was performed using DESeq2 version 1.4.5 (Love et al., 2014). The differential genes with a fold-change (FC) of ≥ 1 and an adjusted P value < 0.05 were classified as differentially expressed genes (DEGs). The Phyper program in R software was used to conduct Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs. The GO terms and KEGG pathways with P < 0.05 were considered significantly enriched. All sequencing data have been deposited in the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (NCBI) database under the accession number GSE293812.

5.4.7 Validation by real-time PCR

The RNA was transcribed into complementary DNA (cDNA) with the SweScript All-in-One RT SuperMix (G3337, Servicebio Technology Co., Ltd., Wuhan, China), following the manufacturer's guidelines. Primers were designed using Primer3 software (https://primer3.ut.ee/) and synthesized by Servicebio Technology Co., Ltd. (Wuhan, China) (**Table 5.1**). A total of 7 were selected DEGs in the same RNA samples were evaluated by quantitative polymerase chain reaction (qPCR) to verify the reliability and reproducibility of RNA-seq. These DEGs were Beta-1,4-N-Acetyl-Galactosaminyl transferase 2 (B4GALNT2), Fatty Acid Binding Protein 1 (FABP1), Glutathione S-Transferase Alpha 1 (GSTA1), Paired Box 9 (PAX9), Paired Box 5 (PAX5), Fc Receptor Like 4 (FCRL4), and Fc Receptor Like A (FCRLA). The qPCR was carried out on the CFX Connect™ Real-Time PCR System (Bio-Rad, California, USA) using a 15 μL reaction mixture containing 2 μL of cDNA, 1.5 μL of each primer (10 μM), 7.5 μL of 2× Universal Blue SYBR Green qPCR Master Mix (G3326, Servicebio Technology Co., Ltd., Wuhan, China), and 4 μL of nuclease-free water. The thermal cycling

protocol included an initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 60°C for 30 seconds. Relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method (Love et al., 2014), with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal reference. The results converted into fold change (FC) values for comparison with RNA-Seq data.

Table 5.1 Primer sequences used in quantitative PCR.

Gene	Primer sequences	Accession No.
B4GALNT2	(F): TGA CCA ACT TCG CCA GAA CA	508108
	(R): TTC CGC TCT GTT GAA ACG GT	
FABP1	(F): GTA CCA AGT CCA GAC CCA GG	327700
	(R): GAT TTC CGA CAC CCC CTT GAT	
GSTA1	(F): GGA GAC AGA CTT TCG TAA GGA TTG A	777644
	(R): ATC TTT TTC AGC AGG TGG GCA	
PAX9	(F): GTG GTC CCA ACA CTC CCT TT	540196
	(R): CAT GAT TCA CCG AGA GCC CA	
PAX5	(F): CGA AGA ACA CGG ACA CCC TT	538371
	(R): AAG AGC TTC TCG CCA TGT GA	
FCRL4	(F): CCA GAC ACT CGG CTT CAC TT	534753
	(R): CAG TGT TTC AGC ACC ACA CG	
FCRLA	(F): AGC AGC CAC TGA GGA CAA TC	782871
	(R): CTA ACA GTA TAC CAG GGG CAG T	

Abbreviations: B4GALNT2, Beta-1,4-N-Acetyl-Galactosaminyltransferase 2; FABP1, Fatty Acid Binding Protein 1; GSTA1, Glutathione S-Transferase Alpha 1; PAX9, Paired Box 9; PAX5, Paired Box 5; FCRL4, Fc Receptor Like 4; FCRLA, Fc Receptor Like A.

5.4.8 Data and statistical analysis

Normally of data distribution was confirmed using the Shapiro-Wilk procedure of SPSS (version 27, Chicago, IL, USA). The data on plasma biochemical

parameters were analyzed using the General Linear Model (GLM) in SPSS to analyze variables that were repeatedly measured over time. Sphericity was assessed using Mauchly's test, with the Greenhouse-Geisser correction applied whenever the assumption was breached. The significance level was set a P value < 0.05.

5.5 Results

5.5.1 Quality of RNA-seq reads

A comparative RNA-seq analysis was performed on the jejunal epithelium transcriptome to evaluate the effects of postbiotic LIC37 supplementation on the transcriptomic profile of the jejunal epithelium in calves. The RNA-seq results for jejunal epithelium samples are presented in **Table S5.1**. The raw data reads ranged from 45.44 million to 47.19 million, with an average of 46.10 million reads. After filtering out low-quality reads, contamination, and other artifacts from the raw data, the number of clean reads ranged from 43.87 million to 45.15 million, with an average of 44.43 million. The GC content of clean reads varied from 49.26% to 52.10%, averaging 50.52%. Additionally, at least 95.70% of the reads had a sequence quality score greater than Q30 (percentage of bases with a Phred value ≥ 30). High-quality reads were mapped to the reference genome at a ratio of 97.11% to 98.04%, with an average of 97.53%.

5.5.2 DEGs Analysis

Eight cDNA libraries were constructed to identify DEGs associated with the postbiotic LIC37 response in the jejunum. As illustrated in **Figure 5.1**, a total of 76 DEGs were identified, comprising 36 upregulated and 40 downregulated genes.

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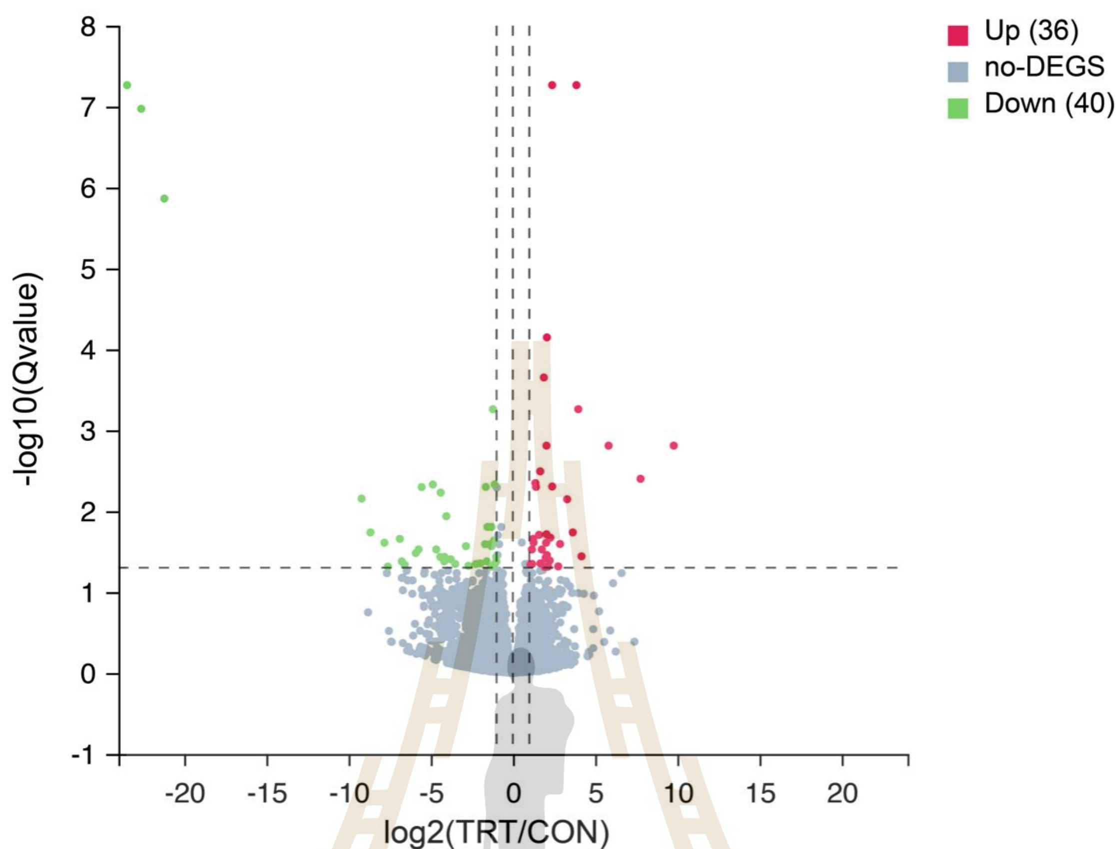

Figure 5.1 Volcano plot of differentially expressed genes in the jejunal tissue of calves. The genes meeting the conditions of adjusted P (Q value) < 0.05 and |log2 FC| ≥ 1 are considered as significant differentially expressed genes (DEGs), with red and green dots representing upregulated and downregulated transcripts, respectively. Gray dots represent insignificant DEGs. The x and y axes of the volcano plots show the log2 fold changes and –log10 q value, respectively.

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The genes related to metabolism and immunity were regulated by postbiotic LIC37 supplementation, such as Fatty Acid Binding Protein 1 (FABP1), and Glucosylceramidase Beta 3 (GBA3). Additionally, the genes associated with intestinal barrier and immunity, such as Beta-1,4-N-Acetyl-Galactosaminyltransferase 2 (B4GALNT2), Glutathione S-Transferase A1 (GSTA1), and One Cut Homeobox 2 (ONECUT2). On the other hand, the genes associated with inflammation showed a downregulation, such as Paired Box 5 (PAX5), Paired Box 9 (PAX9,), Fc Receptor Like A

(FCRLA), and Fc Receptor Like 4 (FCRL4). The top 20 upregulated and top 20 downregulated DEGs were presented in **Table 5.2**.

Table 5.2 Top 20 upregulated and downregulated differentially expressed genes affected by postbiotic LIC37 supplementation in the jejunal tissue of calves.

Gene ID	Symbol[1]	Log2 fold change	Qvalue[2]	Regulated
508108	B4GALNT2	9.7874	1.55E-03	Up
327700	FABP1	7.7692	3.97E-03	Up
539625	GBA3	5.8228	1.55E-03	Up
786706	-	4.1697	3.61E-02	Up
777644	GSTA1	3.9776	5.49E-04	Up
539937	ARL14	3.8660	5.44E-08	Up
782542	ONECUT2	3.6487	1.83E-02	Up
511869	TM4SF5	3.2932	7.12E-03	Up
505865	FOLH1B	2.8627	2.54E-02	Up
538670	FAM151A	2.7599	4.83E-02	Up
414732	GATM	2.3843	4.97E-03	Up
525682	NOTUM	2.3823	5.44E-08	Up
786760	BTN3A3	2.2747	2.13E-02	Up
514667	MST1	2.2436	4.08E-02	Up
100336768	ROS1	2.1404	4.85E-02	Up
407225	MOGAT1	2.0725	3.49E-02	Up
511097	SLC46A1	2.0696	7.13E-05	Up
100300004	GLTPD2	2.0471	1.55E-03	Up
282605	FAM13A	2.0424	1.92E-02	Up

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Table 5.2 Continue.

Gene ID	Symbol[1]	Log2 fold change	Qvalue[2]	Regulated
513137	TMEM72	2.0322	3.76E-02	Up
540196	PAX9	-23.4653	5.44E-08	Down
112444345	-	-22.6113	1.07E-07	Down
112446673	-	-21.2060	1.37E-06	Down
538371	PAX5	-9.2089	6.98E-03	Down
534753	FCRL4	-8.6701	1.83E-02	Down
782871	FCRLA	-7.8139	2.45E-02	Down
531420	GP2	-7.6012	4.85E-02	Down
504258	SIGLEC10	-6.8829	2.20E-02	Down
407126	CR2	-6.7550	4.18E-02	Down
107131854	-	-6.5830	4.60E-02	Down
408008	KCNN1	-5.9122	3.30E-02	Down
512439	HBA	-5.7393	2.97E-02	Down
112445446	-	-5.5555	5.03E-03	Down
493988	SLC14A1	-4.8759	4.67E-03	Down
101908107	P2RY8	-4.6693	2.97E-02	Down
515911	STRA6	-4.4179	3.68E-02	Down
782922	-	-4.3880	5.88E-03	Down
616320	SLC9B2	-4.1830	4.18E-02	Down
100296105	-	-4.1574	3.68E-02	Down
506550	TSPAN1	-4.0508	1.16E-02	Down

[1] B4GALNT2, Beta-1,4-N-Acetyl-Galactosaminyl transferase 2; FABP1, Fatty Acid Binding

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Protein 1; GBA3, Glucosylceramidase Beta 3; GSTA1, Glutathione S-Transferase Alpha 1; ARL14, ADP Ribosylation Factor Like GTPase 14; ONECUT2, One Cut Homeobox 2; TM4SF5, Transmembrane 4 L Six Family Member 5; FOLH1B, Folate Hydrolase 1B; FAM151A, Family with Sequence Similarity 151 Member A; GATM, Glycine Amidino transferase; NOTUM, Notum, Palmitoleoyl-Protein Carboxylesterase; BTN3A3, butyrophilin subfamily 3 member A3; MST1, Macrophage Stimulating 1; ROS1, Receptor Tyrosine Kinase 1; MOGAT1, Monoglyceride O-acyltransferase 1; SLC46A1, Solute Carrier Family 46 Member 1; GLTPD2, GLTP Domain Containing 2; FAM13A, Family with Sequence Similarity 13 Member A; TMEM72, Transmembrane Protein 72; PAX9, Paired Box 9; PAX5, Paired Box 5; FCRL4, Fc Receptor Like 4; GP2, Glycoprotein 2; FCRLA, Fc Receptor Like A; GP2, Glycoprotein 2; SIGLEC10, Sialic Acid Binding Ig-like Lectin 10; CR2, Complement Receptor 2; KCNN1, Potassium Channel, Calcium Activated, Subfamily N, Member 1; HBA, Hemoglobin Subunit Alpha; SLC14A1, Solute Carrier Family 14 Member 1; P2RY8, Purinergic Receptor P2Y8; STRA6, Stimulated by Retinoic Acid 6; SLC9B2, Solute Carrier Family 9 Member B2; TSPAN1, Tetraspanin 1.

[2] Q value is adjusted P value.

5.5.3 GO and KEGG enrichment analysis of DEGs

GO and KEGG pathway analyses were performed on the 76 identified DEGs. The GO analysis classified these DEGs into three categories: biological processes (BP), molecular functions (MF), and cellular components (CC). A total of 352 enriched GO terms were identified. In the BP category, 152 GO terms were significantly enriched. The five most significant GO terms included mitral valve formation (P = 4.39E-05), positive regulation of metallopeptidase activity (P = 8.76E-05), prostaglandin metabolic process (P = 3.88E-04), reproductive process (P = 4.05E-04), and post-anal tail morphogenesis (P = 0.0017). For the MF category, 53 GO terms showed significantly enriched. Among these, the top five GO terms were transmembrane signaling receptor activity (P = 0.0065), actin filament binding (P = 0.0038), molecular function (P = 0.0200), extracellular matrix structural constituent (P = 0.0013), and peroxidase activity (P = 0.0084). In the CC category, 20 GO terms were significantly enriched. The top five most significant GO terms included membrane (P = 0.0031), cellular component (P = 0.014), integral component of Golgi membrane (P = 0.0164), extracellular space (P = 0.0165),

and integral component of plasma membrane (P = 0.0189). **Figure 5.2** presents the 20 most significantly enriched GO terms.

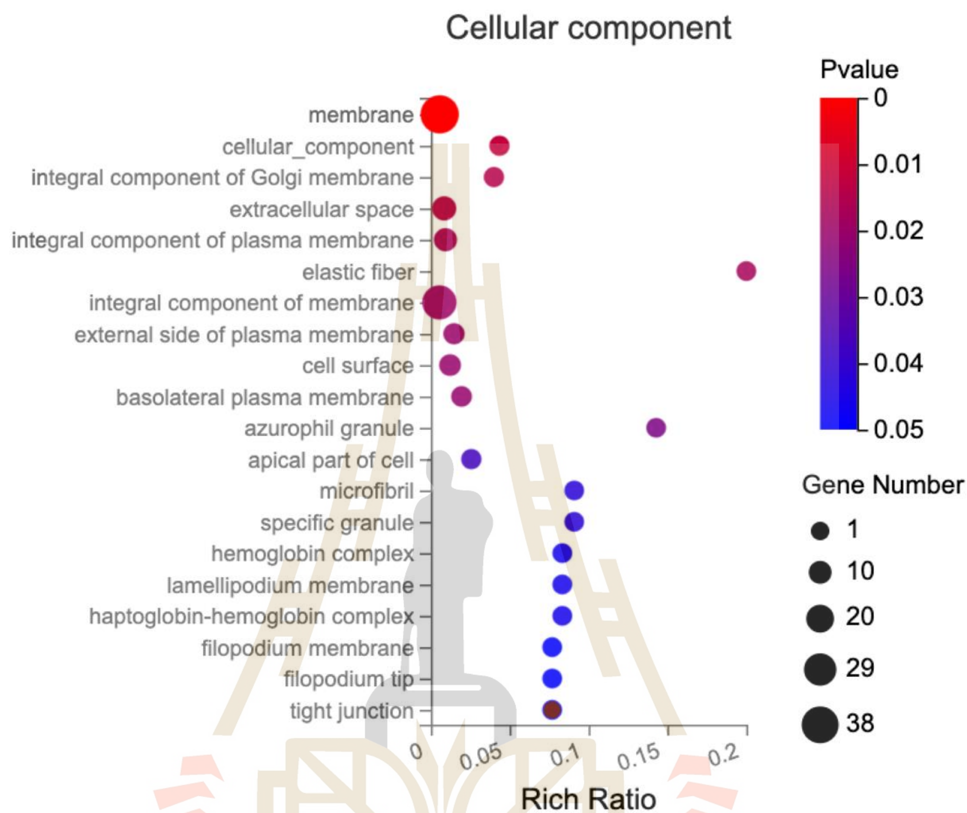

Figure 5.2 Top 20 GO cellular component terms enriched in differentially expressed genes in jejunal epithelium in calves. The circle size in each term corresponds to the number of genes. The circle's color goes from blue to red, indicating a lower P value.

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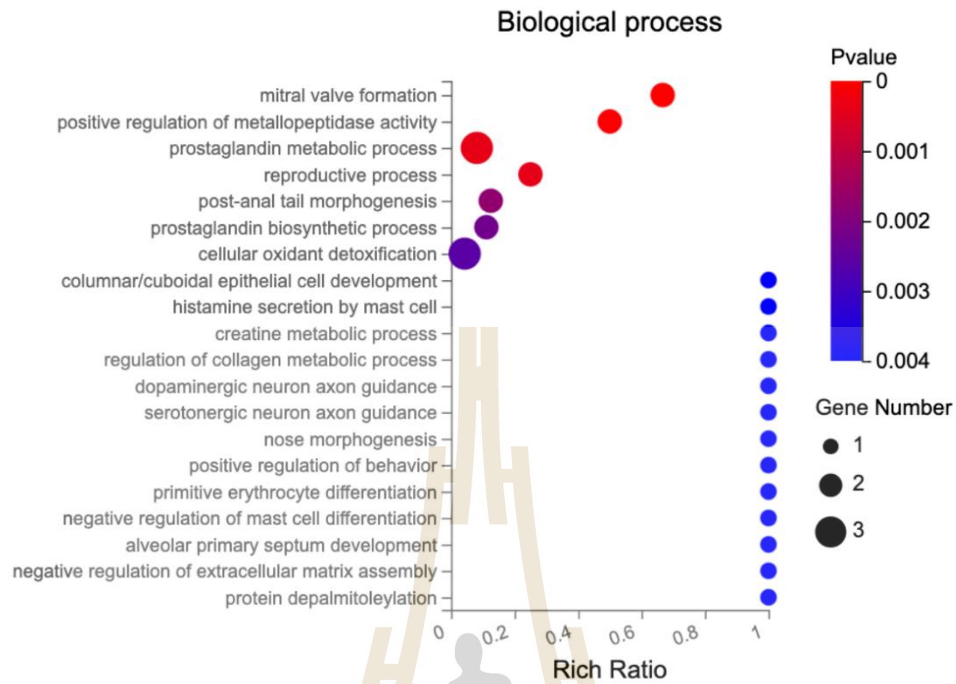

Figure 5.3 Top 20 GO biological process terms enriched in differentially expressed genes in jejunal epithelium in calves.

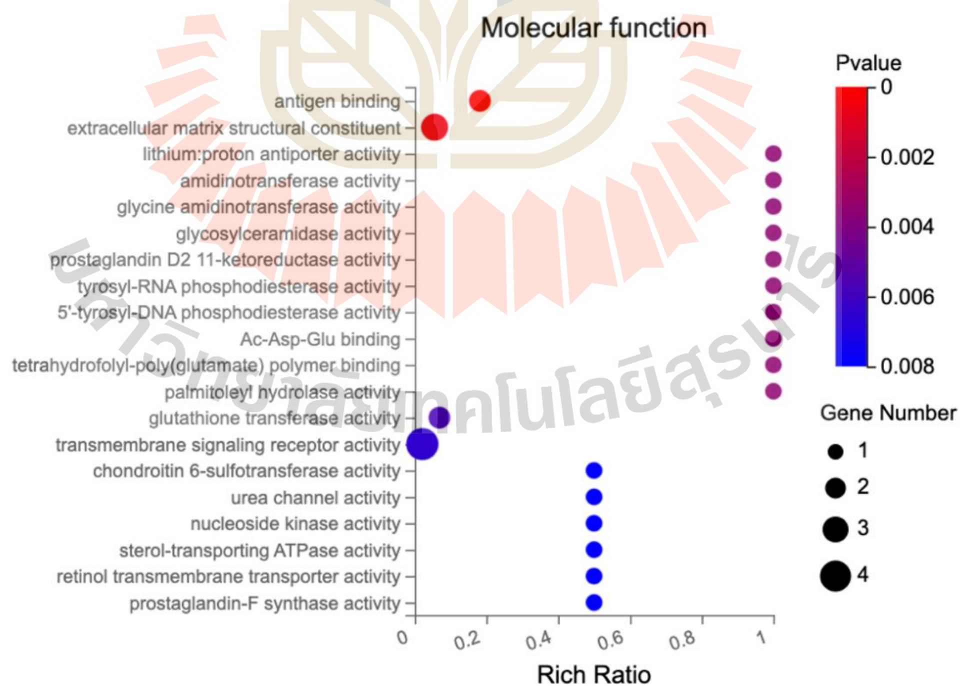

Figure 5.4 Top 20 GO molecular function terms enriched in differentially expressed genes in jejunal epithelium in calves.

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KEGG pathway analysis of the jejunal epithelial tissue identified 8 significantly enriched pathways, namely Arachidonic acid metabolism (P = 0.0045), Drug metabolism - other enzymes (P = 0.0020; bta00983), Vitamin digestion and absorption (P = 0.0036; bta04977), Glutathione metabolism (P = 0.0182; bta00480), Drug metabolism - cytochrome P450 (P = 0.0182; bta00982), Chemical carcinogenesis - DNA adducts (P = 0.0199; bta05204), Metabolism of xenobiotics by cytochrome P450 (P = 0.0211; bta00980), Hepatocellular carcinoma (P = 0.0198; bta05225), and Platinum drug resistance (P = 0.0280; bta01524). **Figure 5.3** presents the 20 most enriched KEGG pathways.

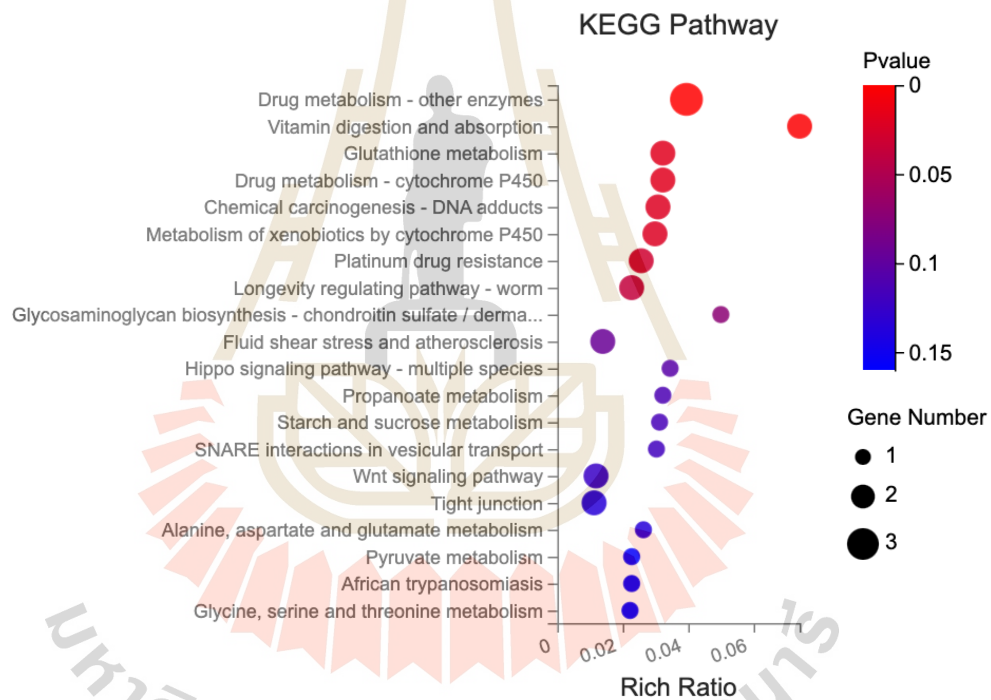

Figure 5.5 Top 20 enriched KEGG pathway of differentially expressed genes in jejunal tissue of calves. The circle size in each term corresponds to the number of genes. The circle's color goes from blue to red, indicating a lower P value.

5.5.4 Validation of RNA-seq results by quantitative PCR

To validate the RNA-seq results, we selected a subset of DEGs for qPCR analysis. Specifically, the expression levels of seven DEGs, including three upregulated

genes (e.g. B4GALNT2, FABP1, and GSTA1) and four downregulated genes (e.g. PAX9, PAX5, FCRL4, and FCRLA), were quantified in jejunal tissue. The qPCR results exhibited expression patterns consistent with the RNA-seq data, confirming the reliability and accuracy of our transcriptomic analysis (**Figure 5.4**).

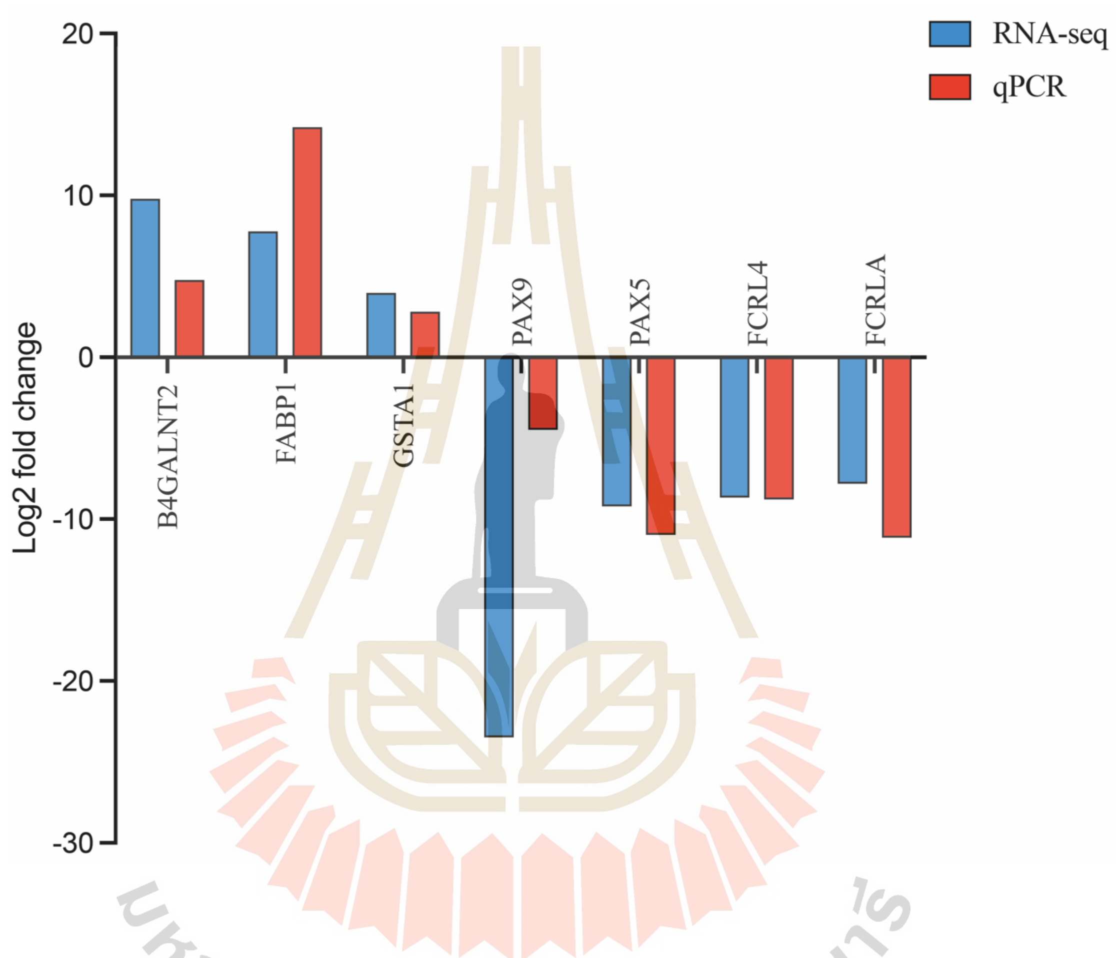

Figure 5.6 Quantitative PCR (qPCR) was employed to validate five DEGs in RNA-seq analysis. The x-axis denotes the genes, while the y-axis shows their mRNA expression levels as fold-change (FC) values. Expression levels obtained from RNA-seq and qPCR are illustrated by blue and red bars, respectively. B4GALNT2, Beta-1,4-N-Acetyl-Galactosaminyl transferase 2; FABP1, Fatty Acid Binding Protein 1; GSTA1, Glutathione S-Transferase Alpha 1; PAX9, Paired Box 9; PAX5, Paired Box 5; FCRL4, Fc Receptor Like 4; FCRLA, Fc Receptor Like A.

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5.6 Discussion

In ruminants, the rumen is not fully developed before weaning, making the small intestine the primary organ responsible for nutrient absorption and immune function (Song et al., 2021). A major function of the intestinal epithelium is to transport and present dietary and bacterial antigens to the immune system (Kelly and Coutts, 2000). The transcriptome of the small intestinal epithelium may provide insights into key genes involved in the regulation of nutrient metabolism and immune function. Postbiotics consist of metabolites such as bacteriocins, peptides, organic acids, and volatile compounds, which display antioxidant and antimicrobial properties (Vasiee et al., 2020; Amiri et al., 2022). Furthermore, they may aid in preventing pathogen adhesion to the gut (Reuben et al., 2020), thereby acting as protectors of intestinal health. In the present study, we identified multiple genes associated with immune response and inflammation, metabolism and detoxification, and cellular signaling.

Of which, FABP1 was highly expressed in bovine jejunal epithelial and involved in fatty acid metabolism (Hayashi et al., 2013), which exerts a cytoprotective role by binding toxic molecules like free fatty acids and heme (Wang et al., 2015), while its antioxidant activity, driven by methionine and cysteine residues, helps reduce reactive oxygen species (ROS) production (Yan et al., 2009). Previous studies have reported that downregulated expression of the FABP1 in the intestine of broilers under stress, such as heat stress (Habashy et al., 2017), and high stocking density (Elbaz et al., 2024a). Consistent findings have reported in monogastric animals. For instance, Wang et al. (2021) reported that feeding broilers with *Lactobacillus plantarum* 16 significantly upregulated mRNA expression of FABP1 in the ileal mucosa, which resulted in better transport and absorption of nutrients. Similarly, an increased expression of FABP1 and CAT1 genes were observed in ileal mucosa of broilers fed with *Bacillus subtilis* (Elbaz et al., 2024b). In the present study, we observed that FABP1 gene was significantly upregulated in the TRT group compared with the control group, suggesting that postbiotic LIC37 enhances FABP1 expression to bind toxic macromolecules while simultaneously inhibiting reactive oxygen species production, thereby providing comprehensive protection to intestinal epithelial cells. B4GALNT2, a glycosyltransferase responsible for synthesizing Sd(a)/Cad antigen-like structures (Byrne et al., 2018), which enhances intestinal barrier function, infection resistance, and immune homeostasis

through glycosylation regulation, playing a crucial role in maintaining gut health (CAPON et al., 2001). In the present study, the expression of B4GALNT2 significantly upregulated in the TRT group with a 7.44 of fold change compared to the CON group. This upregulation was indicative of this postbiotic LIC37 promoting the synthesis of numerous carbohydrate structures required for building Sd(a)/Cad-antigen-like structures, which confer protection to the jejunal epithelium. Similarly, Jiang et al. (2023) reported that feeding lactating cows with *Saccharomyces cerevisiae fermentation product* upregulated expression of B4GALNT2, thereby enhancing the ileum's ability to defend against harmful molecules or microorganisms. We observed that postbiotic LIC37 supplementation leads to an upregulation of GBA3 expression in the jejunal epithelium of calves. GBA3 is an enzyme with broad substrate specificity, capable of hydrolyzing various plant-derived β-glucosides, including phenolic glucosides, cyanogenic glucosides, isoflavones, and flavones (Lopes-Marques et al., 2020). The upregulation of GBA3 indicates an enhanced capacity of the intestine to process dietary plant glucosides (Németh et al., 2003). These glucosides may be converted into more absorbable forms through hydrolysis and removal of their sugar moieties, thereby reducing their toxic effects on the host (Liu et al., 2017). These findings indicate that postbiotic LIC37 supplementation may promote the absorption of these nutrients and reduce their potential toxic impact on the host by upregulating GBA3, which enhances the intestinal detoxification and metabolic capacity for these dietary glucosides. GSTA1 exhibits GSH-dependent steroid isomerase activity as well as GSH-dependent selenium independent peroxidase activity (McIlwain et al., 2006), It has been demonstrated to protect cells from the detrimental effects of ROS-induced lipid peroxidation during oxidative stress induced by various factors (Yang et al., 2001). GSTA1 also protects cells by binding to GSH and mitigating oxidative stress, thereby reducing subsequent lipid peroxidation (Sharma et al., 2004). Additionally, a key function of GSTA1 is its role in inhibiting stress signaling kinases (JNK), which in turn influences the activation of caspases and the apoptosis cascade within the cell (Romero et al., 2006). A previous study reported that treatment with antioxidants can reduced the apoptosis of porcine enterocytes by up-regulating the expression of GSTA1 and regulating glutathione related redox homeostasis (Liu et al., 2018). Consistently, postbiotic LIC37 upregulated expression of GSTA1 in the jejunal epithelium of calves. This upregulation of GSTA1 may contribute to the protection of

the intestinal epithelium by enhancing cellular antioxidant defenses and mitigating oxidative stress. Specifically, by increasing GSTA1 levels, postbiotic LIC37 supplementation may reduce oxidative damage, limit inflammation, and prevent cell apoptosis, ultimately supporting the integrity and function of the jejunal epithelium under stress conditions. In the present study, we observed that postbiotic LIC37 supplementation enhances the expression of ONECUT2 in the jejunal epithelium of calves. This finding is consistent with previous research indicating the crucial role of ONECUT2 in regulating cell proliferation, migration, adhesion, differentiation, and metabolism across various tissues such as the liver, pancreas, retina, neurons, and the immune system (Yu et al., 2020; Zhang et al., 2024). In particular, ONECUT2 has been shown to be essential for the development and differentiation of cells in these tissues. In ONECUT2 knockout mice, the lack of this gene led to failure to thrive during the critical period before weaning, with a 25-30% reduction in size and weight by postnatal day 19 and a higher mortality rate (only 70% survived before weaning) (Dusing et al., 2010). These findings highlight the importance of ONECUT2 in early development and suggest that postbiotic LIC37 supplementation may support the jejunal growth and differentiation of calves, potentially enhancing stress resistance during the weaning period. By upregulating ONECUT2, postbiotic LIC37 supplementation could contribute to the maintenance of intestinal barrier integrity, enhancing epithelial cell proliferation and differentiation, and reducing stress-related damage in the jejunum. Similarly, a reported by Jiang et al. (2023), who demonstrated that feeing lactating cows with *Saccharomyces cerevisiae* fermentation product showed a significant upregulated expression of ONECUT2 in ileal epithelium.

On the other hand, the expressions of PAX5 and PAX9 were downregulated by postbiotic LIC37 supplementation. PAX5 and PAX9 are members belonging to the Pax gene family, which are involved in regulating various biological processes (Blake and Ziman, 2014). Specifically, PAX5 has been shown to inhibit several biological activities of B cells, including cell-cell communication, cell adhesion, cellular metabolism, migration, and nuclear processes (Delogu et al., 2006). The downregulation of PAX5 may increase PTEN expression and inhibit the PI3K-AKT signaling pathway, thereby reducing the secretion of TNF-α and IL-6 (Calderón et al., 2021). Regarding PAX9, while its role has been primarily studied in skeletal development (Rodrigo et al., 2003; Borges

et al., 2025), recent findings suggest a potential interaction between PAX9 and the NF-κB pathway (Yu et al., 2019), which is involved in oxidative stress, immune responses and inflammation in the intestine (Ma and Hottiger, 2016). Therefore, the downregulation of PAX5 and PAX9 by postbiotic LIC37 supplementation could further contribute to reducing inflammation and promoting intestinal health by modulating immune-related pathways. We also observed that both FCRLA and FCRL4 genes were downregulated by postbiotic LIC37 supplementation, which may imply that postbiotic LIC37 supplementation could potentially help modulate immune responses by reducing the expression of these Fc receptor-like family members (Bovo et al., 2024). Given that FCRLA and FCRL4 are involved in the regulation of immune function and differentiation of B cells, the downregulation of these genes might be beneficial in attenuating inflammation and potentially promoting immune homeostasis (Sohn et al., 2011; Liu et al., 2024). Specifically, FCRL4 has been shown to act as a molecular switch in B cells, inhibiting adaptive immune signaling (such as BCR signaling) while enhancing innate immune signaling (e.g., TLR9 signaling) (Sohn et al., 2011; Mahata et al., 2019). Therefore, postbiotic LIC37-induced downregulation of FCRLA and FCRL4 could contribute to a shift toward a more balanced immune response, potentially promoting a less pro-inflammatory environment and supporting intestinal health.

GO analysis provides valuable insights into gene functions, elucidating key biological processes, molecular functions, and cellular components. In the present study, we identified several GO terms associated with intestinal barrier integrity and development, including Columnar/cuboidal epithelial cell development, Negative regulation of extracellular matrix assembly, Regulation of collagen metabolic process, and Tight junction. These findings suggest that postbiotic LIC37 supplementation may reduce immune activation induced by weaning stress. Additionally, GO terms related to antioxidant capacity, such as Cellular oxidant detoxification, Glutathione metabolic process, Glutathione transferase activity, and Peroxidase activity, indicate that postbiotic LIC37 supplementation may enhance oxidative detoxification, thereby improving intestinal cell survival and reducing free radical damage. Furthermore, we identified GO terms associated with metabolic processes, including Creatine metabolic process, Creatine biosynthetic process, Regulation of phosphate transport, and Sterol-

transporting ATPase activity, suggesting that postbiotic LIC37 supplementation may optimize nutrient absorption through these pathways.

In the present study, we used postbiotic LIC37 to alleviate the inflammatory response in intestinal tract of calves and speculated that it would significantly affect immune response in the jejunum. We found that the DEGs were significantly enriched in the Drug metabolism - other enzymes, Vitamin digestion and absorption, Glutathione metabolism, Drug metabolism - cytochrome P450, Hepatocellular carcinoma, Chemical carcinogenesis - DNA adducts, and Metabolism of xenobiotics by cytochrome P450. Among these, Cytochrome P450-related pathways are mainly responsible for the biotransformation of exogenous compounds (Ferguson and Tyndale, 2011). Glutathione (GSH) serves as a crucial antioxidant and detoxifying molecule, playing a key role in cellular defense, heavy metal chelation, and chemical detoxification (Masella et al., 2005). Likewise, vitamins are essential for immune regulation, antioxidant protection, and energy metabolism (Pisoschi et al., 2022). Similar findings have been reported in previous research. For instance, in a transcriptome study of the ileal epithelium of lactating cows fed *Saccharomyces cerevisiae* fermentation product, significantly enrichment was also observed in Drug metabolism - other enzymes, Glutathione metabolism, and Drug metabolism - cytochrome P450 (Jiang et al., 2023). Similarly, Zhang et al. (2017) found that feeding weaned piglets with *Lactobacillus* led to significant enrichment in Arachidonic acid metabolism, Vitamin digestion and absorption, and Metabolism of xenobiotics by cytochrome P450. Consistently, our study found that dietary supplementation with postbiotic LIC37 influenced similar pathways, suggesting that postbiotic LIC37 may modulate intestinal homeostasis by regulating detoxification, oxidative stress defense, and nutrient metabolism.

5.7 Conclusion

This study demonstrates that dietary supplementation with postbiotics from heat killed *Limosilactobacillus ingluviei* C37 positively influence intestinal health in calves. Postbiotic LIC37 supplementation reduced globulin, and neutrophil levels, indicating its potential to mitigate systemic inflammation. Transcriptomic analysis revealed that postbiotic LIC37 modulates key pathways involved in nutrient metabolism, oxidative stress defense, and immune regulation by upregulating genes

such as FABP1, B4GALNT2, and GSTA1, while downregulating pro-inflammatory genes like PAX5 and PAX9. These findings suggest that postbiotic LIC37 enhance intestinal barrier function, mitigate oxidative stress, and promote immune homeostasis, thereby supporting calf health during the critical weaning period. Thus, postbiotic LIC37 represent a promising and sustainable feed additive with broad applications in ruminant nutrition.

5.8 References

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CHAPTER VI
OVERALL CONCLUSION AND IMPLICATION

6.1 Conclusion

The use of postbiotics as a novel alternative to probiotic in animal nutrition has gained increasing interest due to their safety and stability. Postbiotics have been reported to exert beneficial effects, including modulation of gut microbiota, enhancement of antioxidant capacity, and improvement of host metabolism and immunity. Therefore, this study investigated the effects of supplementation of postbiotic from heat killed *Limosilactobacillus ingluviei* C37 (postbiotic LIC37) on growth performance, antioxidant capacity, and transcriptomic responses in calves during weaning. The main findings are summarized as follows:

Postbiotic LIC37 supplementation improved feed efficiency without affecting body weight or feed intake. It also modulated systemic immunity and metabolism by reducing serum globulin, total protein, neutrophil count, and neutrophil-to-lymphocyte ratio, alongside tendencies toward decreased BUN, triglyceride, and cholesterol levels. In terms of antioxidant status, postbiotic LIC37 decreased cortisol and MDA levels while enhancing CAT activity and showing trends of increased GPX levels and DPPH scavenging capacity. Transcriptomic profiling of the liver and jejunum further indicated regulatory effects on lipid metabolism, antioxidant defense, intestinal barrier function, and detoxification, notably through the activation of PPAR signaling, glutathione metabolism, and drug metabolism pathways.

Together, these findings suggest that postbiotics from heat killed *Limosilactobacillus ingluviei* C37 may enhance metabolic efficiency, alleviate oxidative stress, and improve gut health in calves during the weaning period.

6.2 Implication

This study demonstrated that postbiotic from heat killed *Limosilactobacillus ingluviei* C37 has the potential to improve feed efficiency, antioxidant capacity, immune

regulation, and gut microbial composition in calves during the weaning period. Unlike conventional probiotics, postbiotics are more stable under harsh environmental conditions such as high temperature and pressure during feed processing. This stability offers practical advantages for large-scale application in ruminant nutrition. Further research is warranted to explore optimal dosages, delivery forms, and long-term impacts on calf development, as well as to evaluate the efficacy of postbiotics across different breeds and feeding systems.

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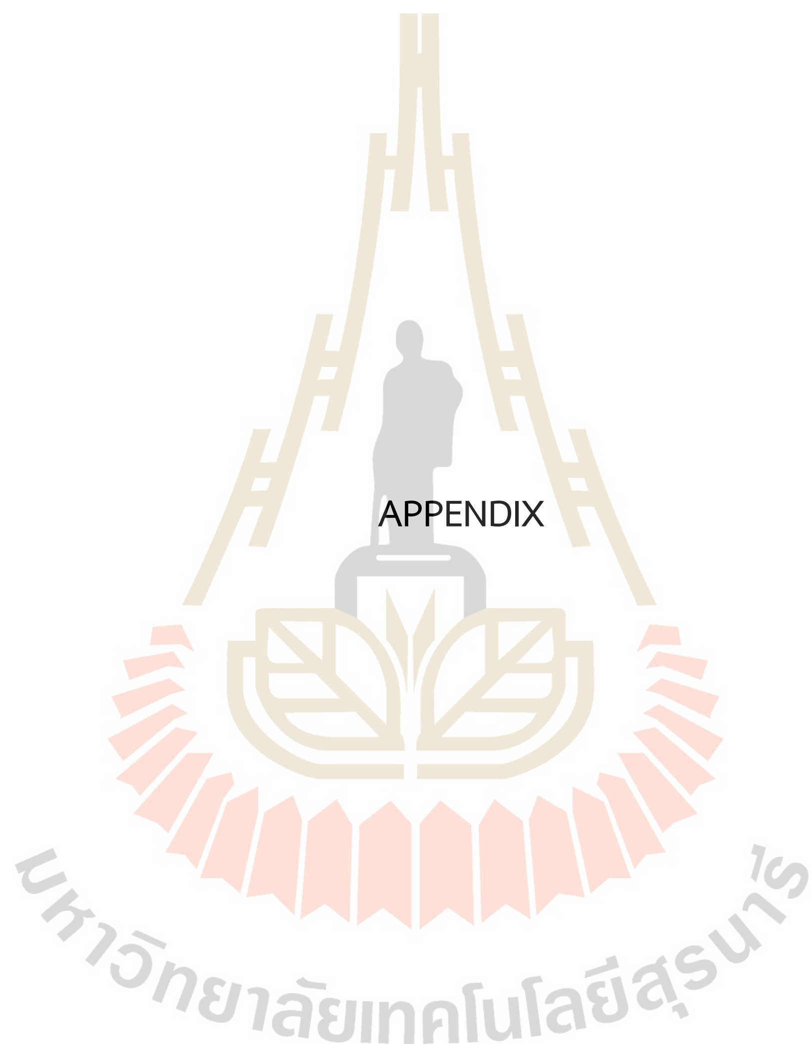
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APPENDIX

QR code for Supplementary files

Figure S 4.1 Curve of amplification of DEGs for qPCR

Figure S 4.2 Curve of dissolution of DEGs for qPCR

Table S 4.1 RNA-sequencing reads and mapping rates in the liver tissue of calves

Table S 4.2. Differentially expressed genes in the liver tissue of calves

Table S 4.3 Gene Ontology analysis of DEGs in liver tissue in calves

Table S 4.4 Kyoto encyclopedia of genes and genomes pathways possibly affected by postbiotic in liver tissue in calves

Figure S 5.1 Curve of amplification of DEGs for qPCR

Figure S 5.2 Curve of dissolution of DEGs for qPCR

Table S 5.1 RNA-sequencing reads and mapping rates in the jejunal tissue in calves

Table S 5.2. Differentially expressed genes in the jejunal epithelium of calves

Table S 5.3 Gene Ontology (GO) analysis of DEGs in jejunal epithelium in calves

Table S 5.4 Kyoto encyclopedia of genes and genomes pathways possibly affected by postbiotic in jejunal epithelium in calves

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BIOGRAPHY

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